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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68, 1/70, C12P 19/34, C07H 21/02, 21/04, G01N 33/53, C07K 15/28, 5/00	A1	(11) International Publication Number: WO 97/17470 (43) International Publication Date: 15 May 1997 (15.05.97)
(21) International Application Number: PCT/US96/17877 (22) International Filing Date: 8 November 1996 (08.11.96) (30) Priority Data: 08/555,394 9 November 1995 (09.11.95) US (71)(72) Applicant and Inventor: HOLLAND, James, F. [US/US]; 31 Mamaroneck Road, Scarsdale, NY 10583 (US). (72) Inventor: POGO, Beatriz, G., T.; 237 Nyac Avenue, Pelham, NY 10803 (US). (74) Agents: CLARK, Richard, S. et al.; Brumbaugh, Graves, Donohue & Raymond, 30 Rockefeller Plaza, New York, NY 10112 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: DETECTION OF MAMMARY TUMOR VIRUS-LIKE SEQUENCES IN HUMAN BREAST CANCER (57) Abstract The present invention relates to materials and methods for diagnosing breast cancer in humans. It is based, at least in part, on the discovery that a substantial percentage of human breast cancer tissue samples contained nucleic acid sequences corresponding to a portion of the mouse mammary tumor virus <u>env</u> gene. In contrast, such sequences were absent in almost all other human tissues tested.		

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Description

Detection Of Mammary Tumor Virus-Like Sequences In Human Breast Cancer

Cross-Reference to Related Application

This application is a continuation-in-part application of U.S. Serial No. 08/555,394, filed November 9, 1995.

Statement Regarding Federally Sponsored Research

- 5 This invention was made with funds from the U.S. government, which has certain rights in the invention.

Introduction

- 10 The present invention relates to materials and methods for diagnosing breast cancer in humans. It is based, at least in part, on the discovery that a substantial percentage of human breast cancer tissue samples contained nucleic acid sequences corresponding to a portion of the mouse mammary tumor virus env gene. In contrast, such sequences were absent in almost all other human tissues tested.
- 15

Background of the Invention

- 20 A large body of information has accumulated about the molecular biology of MMTV (reviewed in Slagle, B.L. et al., 1987, in "Cellular and Molecular Biology of Mammary Cancer", Kidwell et al., eds., Plenum Press, NY. pp 275-306). Mouse mammary tumor virus (MMTV) is associated with a high incidence of breast cancer in certain strains of mice (over 90% among females), and has been regarded as a potential model for human disease.
- 25

The MMTV virus does not carry a transforming oncogene, but rather acts as an insertional mutagen with several proviral insertion loci designated int-1

or wnt-1 (Nusse R. et al., 1982, Cell 31:99-109) int-2
(Peters, G. et al., 1983, Cell 33:369-377) int-3
(Gallahan, D. et al., 1987, J. Virol. 61:218-220) int-4
(Roelink, H. et al., 1990, Proc. Natl. acad. Sci. USA
5 87:4519-4523) and int-5 (Morris, V.L., et al. 1991,
Oncogene Research 6:53-63), which encode for growth
factors or other related proteins. These genes are not
expressed in normal mammary tissue but become activated
after integration of MMTV provirus into the adjacent
10 chromosomal DNA.

The human homolog of the int-2 locus has been
located on chromosome 11 (Casey, G. et al., 1986,
Mol. Cell Biol. 6:502-510) and has been found amplified
(in 15% of the breast cancers) and also expressed
15 (Lidereau, R. et al., 1988, Oncogene Res 2:285-291;
Zhou, D.J. et al., 1988, Oncogene 2:279-282; Liscia,
D.S. et al., 1989, Oncogene 4:1219-1224; Meyers, S.L.
et al., 1990, Cancer Res 50:5911-5918). It may be
significant that in tumors from Parsi women, who have a
20 high incidence of breast tumors, the int-2 locus is
amplified in 50% of the cases (Barnabas-Sohi, N. et
al., 1993, Breast Dis. 6:13-26). The amplification of
int-2 and other genes in 11q13 is indicative of poor
prognosis (Schuwring, E. et al., 1992, Cancer Research
25 52:5229-5234; Champeme, M-H, et al., 1995, Genes,
Chromosomes and Cancer 12:128-133). Both mouse and
human int-2 have been sequenced (Moore, R. et al.,
1986, EMBO J 5:919-924). The gene encodes a protein of
about 27 kilodaltons (KD) which shows homology to both
30 basic and acidic fibroblast growth factors (Dickson, C.
et al. 1987, Nature (London) 326:833).

However, efforts to demonstrate the presence of
viruses in human breast cancer through search for viral
particles, immunological cross-reactivity, or sequence
35 homology have yielded contradictory results. Detect-
able MMTV env gene-related antigenic reactivity
has been found in tissue sections of breast cancer

(Mesa-Tejada et al., 1978, Proc. Natl. Acad. Sci. USA 75:1529-1533; Levine, P. et al., 1980, Proc. Am. Assoc. Cancer Res. 21:170; Lloyd, R. et al., 1983, Cancer 51:654-661), breast cancer cells in culture (Litvinov, S.V. and Golovkina, T.V., 1989, Acta Virologica 33:137-142), human milk (Zotter S. et al., 1980, Eur. J. Cancer 16:455-467) in sera of patients (Day, N.K. et al., 1981, Proc. Natl. Acad. Sci. USA 78:2483-2487), in cyst fluid (Witkin, S.S. et al., 1981, J. Clin. Invest. 67:216-222) and in particles produced by a human breast carcinoma cell line (Keydar, I. et al., 1984, Proc. Natl. Acad. Sci. USA 81:4188-4192). Sequence homology to MMTV has been found in human DNA under low stringency conditions of hybridization (Callahan, R. et al., 1982, Proc. Natl. Acad. Sci. USA 79:5503-5507) and RNA related to MMTV has been detected in human breast cancer cells (Axel, R. et al., 1972, Nature 235:32-36). The presence of MMTV related sequences in lymphocytes from patients with breast cancer has been reported (Crepin, M. et al., 1984, Biochem. Biophys. Res. Comm. 118:324-331), as well as detection of reverse transcriptase (RT) activity in their monocytes (Al-Sumidaie, A.M. et al., 1988, Lancet 1:5-8). May and Westley (May and Westley, 1989, Cancer Research 49:3879-3883) have reported the presence of MMTV-like sequences arranged as tandem repeats only in DNA from breast cancer cells.

These results have been difficult to interpret, and theories linking MMTV or a related virus with human breast cancer have fallen out of favor, in view of the relatively recent discovery of human endogenous retroviral sequences ("HERs"; Westley, B. et al., 1986, J. Virol. 60:743-749; Ono, M. et al., 1986, J. Virol. 60:589-598; Faff, O. et al., 1992, J. Gen. Virology 73:1087-1097). Data which could be interpreted to demonstrate the presence of MMTV-related sequences could be more readily explained by endogenous human

retroviral sequences. Adding further confusion to the picture, env-gene related antigenicity has been detected in epitopes of human proteins (Hareuveni, M. et al., 1990, Int. J. Cancer 46:1134-1135).

5 Brief Summary of the Invention

 The present invention relates to methods for diagnosing breast cancer in humans in which the presence of mouse mammary tumor virus env gene-like sequences bears a positive correlation to the existence
10 of malignant breast disease. It is based, at least in part, on the discovery that 38 to 40 percent of human breast cancer tissue samples tested contained gene sequences homologous to the mouse mammary tumor virus env gene that are substantially absent from other human
15 tumors and tissues. The invention also relates to methods for diagnosing breast cancer in humans in which the presence of retrovirus proviral fragments substantially homologous to the env gene and/or 3' LTR sequence of MMTV are detected. The molecular probes
20 used in these experiments were designed to avoid cross-hybridization with endogenous human retroviral sequences. The present invention further provides for compositions of molecular probes which may be utilized in such diagnostic methods.

25 Brief Description of the Figures

FIGURE 1: Amplification of 660 bp of MMTV-like env gene. DNA was extracted from frozen tissues. PCR was performed using primers 1 and 3. A: 2% agarose gel electrophoresis. B: Southern blot hybridization
30 using 5',³²P-end-labeled probe 2. Lanes 1 and 3: breast cancer; lanes 2 and 4: normal breast; lane 5: control reaction (no DNA); lane E: MMTV env gene. M: molecular weight marker. Arrow indicates 510 bp band.

FIGURE 2: Nested PCR. A: 2% agarose gel electrophoresis. 1: Amplification of 686 bp of MMTV-like env
35

gene sequences using primers 1 and 4 and the product of reaction A 1 as template. 2: Amplification of 250 bp of MMTV-like env gene sequences using primers 2 and 3. B, 1 and 2: Southern blot hybridization of the amplified products using probe 5'-³²P end-labeled probe 2a.

FIGURE 3: Amplification of 250 bp of MMTV-like env gene. DNA was extracted from paraffin-embedded tissue sections. PCR was performed using primers 2 and 3. A: 2% agarose gel electrophoresis. B: Southern blot hybridization using 5'-³²P-labeled probe 2a. Lane 1: normal breast; lanes 2 to 5: breast cancer; lane E: MMTV env gene. M: molecular weight marker. Arrow indicates 298 bp band.

FIGURE 4: Nucleotide sequence of the cloned MMTV env gene-like sequences as compared to the env sequences of the GR and BR6 strains of MMTV using the GCG program. *:potential glycosylation site, |:mismatch to MMTV.

FIGURE 5: Southern blot hybridization of genomic DNA. DNA was extracted from frozen tissues or cell lines, digested with EcoRI and transferred to nitrocellulose paper. Hybridization with ³²P-labeled clone 166. DNA from A, B, and G: env gene positive breast cancer; C and D: env negative breast cancer; E and F: normal breast; H: MCF-7 cells. M: molecular weight marker, Arrow indicates 9kb band.

FIGURE 6: Southern blot hybridization of genomic DNA. Experimental conditions as in Fig. 5. DNA from A and B: env negative breast cancer; C and D: env positive breast cancer; E: molecular weight marker (non-labelled); F. to H: normal breast. Arrow indicates position of 9 kb marker.

FIGURE 7: Map of MMTV.

FIGURE 8: Comparison of the nucleic acid sequence of mouse mammary tumor env gene ("MMTENV"), showing residues 976-1640, with the nucleic acid sequence of a

representative 660 bp sequence obtained by PCR reaction of DNA from human breast cancer tissue ("MS1627").

FIGURE 9: Sequence of an about 2.6 kb MMTV-like fragment detected in a human breast carcinoma.

5 Detailed Description of the Invention

The present invention relates to methods and compositions for diagnosing breast cancer in humans.

The present invention provides for compositions comprising an isolated and purified nucleic acid
10 molecule which (i) hybridizes to a gene of mouse mammary tumor virus; (ii) is present in at least 20 percent of DNA samples prepared from breast cancer tissue of different human subjects; and (iii) is present in less than 5 percent of DNA samples prepared
15 from tissues other than breast cancer tissue from different human subjects. A "gene of mouse mammary tumor virus" includes, but is not limited to, the gag, pol, and env genes and the 5' LTR and 3' LTR sequences of MMTV. In preferred embodiments of the invention,
20 the mouse mammary tumor virus (hereafter "MMTV") gene is the env gene and/or the 3' LTR sequence. The term "hybridize" is used to refer to routine DNA-DNA or DNA-RNA hybridization techniques under what would be regarded, by the skilled artisan, as stringent
25 hybridization conditions. The phrase "is present" indicates that a native form of the molecule, in an unpurified state (for example, as part of chromosomal DNA), may be detected by a standard laboratory technique, such as Southern blot or polymerase chain
30 reaction (PCR). To be "present", the molecule may be detectable by one technique but not others. To be present in "less than 5 percent of DNA samples prepared from tissues other than breast cancer tissue from different human subjects", all non-breast cancer tissue
35 samples are considered together, but the total number of samples must be large enough to give the 5 percent

value statistical significance that would be reasonable to the skilled artisan.

In order to identify such a nucleic acid molecule, the sequence of MMTV may be compared, using a computer database, to known human DNA sequences, and portions of MMTV which are less than or equal to 25 percent homologous to a human sequence may be selected for further study. The term "homologous", as used herein, refers to the presence of identical residues; for example, a first sequence is considered 25 percent homologous to a second sequence if it shares 25 percent of the residues of the first sequence. Since there is relatively greater likelihood that MMTV may bear similarity to human retroviral-like sequences, it may be preferable to evaluate whether a particular MMTV nucleic acid sequence is homologous to such sequences, for example, as endogenous human retrovirus sequences. A prototype of such viruses is HERV-K10 (Ono, M. et al., 1986, J. Virol. 60:589-598).

Once an MMTV gene sequence which is less than or equal to 25 percent homologous to a human DNA sequence, such as a human endogenous retroviral sequence, is identified, the presence of nucleic acid molecules having the MMTV gene sequence in human breast cancer tissues and other tissues may be evaluated. Such evaluations may be performed either by Southern blot techniques, or, preferably, by polymerase chain reaction (PCR) techniques, which are more sensitive. In such a way, MMTV gene sequences which (i) hybridize to at least 20 percent of DNA samples prepared from breast cancer tissue of different human subjects and (ii) hybridize to less than 5 percent of DNA samples prepared from human tissues other than breast cancer tissues may be identified. A nucleic acid molecule having a MMTV gene sequence which satisfies these requirements may then be used in diagnostic methods which detect the presence of such sequence in human

breast tissue by standard techniques, including PCR techniques which assay for the presence of the molecule, but also, where appropriate, Southern blot, Northern blot, or Western blot techniques, to name but
5 a few.

In preferred embodiments, the present invention relates to a portion of MMTV localized between MMTV env gene sequences 976 and 1640 (Majors, I.E. and Varmus, H.E., 1983, J. Virol. 47:495-504; see Fig. 7). This
10 about 660 bp sequence (hereafter, "the 660 bp sequence") has been found to exhibit low (16 percent) homology to the prototype human endogenous retrovirus HERV-K10, using the IBI/Pustell Sequence Analysis Program, and has also been shown to be present in 121
15 (38.5%) of 314 unselected breast cancer tissue samples, in cultured breast cancer cells, in 2 of 29 breast fibroadenomas (6.9%) and in 2 of 107 breast specimens from reduction mammoplasties (1.8%). The sequence was not found in normal tissues including breast, lympho-
20 cytes from breast cancer patients nor in other human cancers or cell lines (see example section, infra). Similarly, an about 250 bp sequence (hereafter "the 250 bp sequence"), between positions 1388 and 1640 in the env gene, and therefore falling within the 660 bp
25 sequence, was detected in 60 (39.7%) of 151 breast cancer, and in one of 27 normal breast samples assayed from paraffin-embedded sections. Cloning and sequencing of the 660 bp and 250 bp sequences demonstrated that they are 95-99% homologous to MMTV env gene, but
30 not to the known human endogenous retroviruses ("HERs") nor to other viral or human genes (<18%).

In another preferred embodiment, the present invention relates to a nucleic acid molecule which corresponds to a retroviral genomic fragment which has
35 substantial homology to 3' LTR and/or env gene of the MMTV genome, and is found in a substantial percentage of breast cancer samples. By substantial percentage is

meant at least 20% of tested breast cancer samples. Such a sequence is preferably comprised of the 3' LTR region and all or part of the env gene, although it may include more sequences of a retroviral genome. Most preferably, the sequence is at least comprised of an about 2.6 kb fragment which comprises the 1,228 base pair (bp) sequence of the 3' LTR sequence and 1,336 bp of the env gene sequence of MMTV (Fig. 9) (SEQ ID NO:20). When compared with the two strains of MMTV C3H and BR6, the sequence homology was 90.8% and 90.7%, respectively. When compared with the endogenous retroviral sequences (HUMERKA), sequence homology was only 58% in 36 bp and 71% in 74 bp.

Retrovirus proviral sequences can be detected by PCR technology using primers derived from the MMTV genome. Such primers include primer 5L, containing the nucleotides 7376-7395 of the MMTV BR6 genome (5'-3': CCAGATCGCCTTTAAGAAGG) (SEQ ID NO:11) and primer LTR3, containing nucleotides 9918-9927 of the MMTV BR6 genome (5'-3': CGAACAGACACAAAGCGACG) (SEQ ID NO:19). Other primers which correspond to or are homologous to MMTV sequences can be used as primers. Nucleotide fragments which correspond to or are homologous to the retroviral sequences isolated from the breast cancer samples can also be used to amplify additional retroviral fragments from the samples. Long PCR techniques can be used to amplify longer stretches of a proviral sequence.

The present invention provides for compositions comprising an isolated and purified nucleic acid molecule which hybridizes to the about 2.6 kb retroviral fragment shown in Fig. 9 under stringent conditions or is at least 90 percent homologous to said fragment using the MacVector homology determining program which may be used to diagnose breast cancer in a subject, using methods which include PCR and Southern blot methods.

Nucleic acids having the 660 bp sequence, the 250 bp sequence, or all or part of the about 2.6 kb sequence, may therefore be used, according to the invention, to diagnose breast cancer in a subject, using methods which include PCR and Southern blot methods. Where PCR methods are used, primers such as those listed in Table 1, below, may be utilized.

The present invention provides for compositions comprising essentially purified and isolated nucleic acid having the 660 bp sequence or the 250 bp sequence or an at least five bp, and preferably greater than or equal to ten bp, subsequence thereof. In order to maintain the desired specificity, such nucleic acid molecules may preferably contain sequence falling within the 660 bp sequence, but preferably do not contain sequences from other portions of the MMTV genome, which may, undesirably, hybridize to human sequences which are not breast cancer specific, such as HERs. Accordingly, the present invention provides for compositions wherein the isolated and purified nucleic acid molecule comprises at least a portion having a nucleic acid sequence which hybridizes to a region of the mouse mammary tumor virus env gene between residues 976 and 1640, or between residues 1388 and 1640, and wherein the isolated and purified nucleic acid molecule does not hybridize to any other region of the MMTV genome.

The 660 bp sequence, in various embodiments, may have a number of nucleotide sequences. For example, in one embodiment, the 660 bp sequence may have a sequence as set forth in Fig. 8 and designated "MMTENV-like sequence" (SEQ ID NO:17), which depicts the MMTV env sequence between residues 976 and 1640. In a second series of embodiments, the 660 bp sequence may have a sequence as set forth in Fig. 8 and designated "MS1627" (SEQ ID NO:18), which depicts a predominant sequence for the 660 bp sequence as it has been defined by

sequencing analysis of the products of PCR reactions using DNA from human breast cancer tissues. In still further embodiments, the 660 bp sequence may have various other nucleotide sequences obtained by sequencing the results of PCR reactions to detect the presence of 660 bp sequence in human breast cancer tissues.

In related embodiments, the present invention provides for compositions comprising PCR primers that may be used to detect the presence of the forementioned molecules or other MMTV-like sequences. For example, the compositions may comprise one or more of the following primer molecules (5' - 3'):

CCTCACTGCCAGATC (SEQ ID NO:1); GGGAATTCCTCACTGCCAGATC (SEQ ID NO:2); CCTCACTGCCAGATCGCCT (SEQ ID NO:3); TACATCTGCCTGTGTTAC (SEQ ID NO:4); CCTACATCTGCCTGTGTTAC (SEQ ID NO:5); CCGCCATACGTGCTG (SEQ ID NO:6); ATCTGTGGCATACT (SEQ ID NO:7); GGGAATTCATCTGTGGCATACT (SEQ ID NO:8); ATCTGTGGCATACTAAAGG (SEQ ID NO:9); GAATCGCTTGGCTCG (SEQ ID NO:10); CCAGATCGCCTTTAAGAAGG (SEQ ID NO:11); TACAGGTAGCAGCACGTATG (SEQ ID NO:12); CGAACAGACACAAACACACG (SEQ ID NO:19).

The use of such compositions and molecules in PCR and Southern blot techniques is illustrated in the non-limiting examples set forth below. The correlation between the presence of the MMTV-related nucleic acid molecules described above and breast cancer allows such molecules and compositions to be utilized in the diagnosis of breast cancer. Accordingly, the present invention provides for a method of diagnosing breast cancer, wherein the detection of such nucleic acid molecules bears a positive correlation to the existence of breast cancer in a human. The results of such evaluation, together with additional clinical symptoms, signs, and laboratory test values, may be used to formulate the complete diagnosis of the patient.

In further related embodiments, the present invention provides for an essentially purified peptide encoded by a nucleic acid molecule which (i) hybridizes to a gene of MMTV; (ii) is present in at least
5 20 percent of DNA samples prepared from breast cancer tissue of different human subjects; and (iii) is present in less than 5 percent of DNA samples prepared from tissues other than breast cancer tissue from different human subjects. In preferred embodiments, the
10 MMTV gene is the env gene.

Such peptides may be used in the diagnosis of breast cancer. Accordingly, the present invention provides for a method of diagnosing breast cancer in a human subject, comprising detecting the presence of
15 a peptide encoded by a nucleic acid molecule which (i) hybridizes to the env gene of a mouse mammary tumor virus; (ii) is present in at least 20 percent of DNA samples prepared from breast cancer tissue of different human subjects; and (iii) is present in less than
20 5 percent of DNA samples prepared from tissues other than breast cancer tissue from different human subjects.

The present invention also provides for antibodies (including monoclonal and polyclonal) antibodies which
25 specifically bind to such peptides. Such antibodies may be used in methods of diagnosing breast cancer, for example, but not by way of limitation, by Western blot, immunofluorescent techniques, and so forth.

In nonlimiting embodiments of the invention, the
30 skilled artisan may evaluate MMTV-like nucleic acid molecules for regions which would be considered likely to encode immunogenic peptides (using, for example, hydropathy plots). Such peptides may then be sequenced and used to produce antibodies that may be employed in
35 diagnostic methods as set forth above.

For example, certain peptides encoded by portions of the 660 bp sequence have been synthesized. These

peptides, which have the sequences LKRPGFQHEMI (SEQ ID NO:13) and GLPHLIDIEKRG (SEQ ID NO:14), have been used to produce antibodies in rabbits, and the resulting antisera have successfully identified breast cancer cells positive for MMTV env-like sequences by PCR assay. Other peptides encoded by 660 bp sequence which may be useful according to the invention include TNCLDSSAYDTA (SEQ ID NO:15) and DIGDEPWFFDD (SEQ ID NO:16).

10 6. Example: The Detection of Mouse Mammary Tumor Virus Env Gene-Like Sequences in Human Breast Cancer Cells and Tissues

 6.1. Materials and Methods

 DNA from breast cancer tissue and other human cancer tissues, human placentas, normal human tissues including breast, and from several human cell lines (including eight breast cancer cell lines), and two normal breast cell lines was extracted following the procedure of Delli Bovi et al. (1986, Cancer Res. 46:6333-6338). The DNA was resuspended in a solution containing 0.05 M Tris HCl buffer, pH 7.8, and 0.1 mM EDTA, and the amount of DNA recovered was determined by microfluorometry using Hoechst 33258 dye (Cesarone, C. et al., 1979, Anal Biochem 100:188-197). Plasmids containing the cloned genes of MMTV were obtained from the ATCC, propagated in Escherichia coli cultures and purified using anion-exchange minicolumns (Qiagen) or by precipitation with polyethylene glycol (Sambrook J., et al., 1989, in "Molecular Cloning/A Laboratory Manual", Cold Spring Harbor). Oligonucleotide primers were synthesized at the core facilities of the Brookdale Molecular Biology Center at Mount Sinai School of Medicine.

 Polymerase chain reaction (PCR) was performed using Taq polymerase following the conditions recommended by the manufacturer (Perkin Elmer Cetus)

with regard to buffer, Mg^{2+} and nucleotide concentrations. Thermocycling was performed in a DNA cycler by denaturation at 94° C for 3 min. followed by either 35 or 50 cycles of 94° C for 1.5 min., 50° C for 2 min. and 72° C for 3 min. The ability of the PCR to amplify the selected regions of the MMTV env gene was tested by using as positive templates the cloned MMTV env gene and the genomic DNA of the MCF-7 cell line, since it was shown to express gp52 immunological determinants (Yang, N.S., et al., 1975, J. Natl. Cancer Inst. 61:1205-1208). Optimal Mg^{2+} , primer concentrations and requirements for the different cycling temperatures were determined with these templates. The master mix as recommended by the manufacturer was used. To detect possible contamination of the master mix components, a reaction without template was routinely tested. γ DNA and control primers provided by the manufacturer were used as control for polymerase activity. As an internal control, amplification of a 120 bp sequence estrogen receptor gene was assayed using primers designed and generously provided by Dr. Beth Schachter, (Mount Sinai School of Medicine, N.Y.). In addition, primers for actin 5 gene amplification were also used.

The product of the PCR was analyzed by electrophoresis in a 2% agarose gel. A 1 kb DNA ladder (Gibco BRL) was used to identify the size of the PCR product. To determine if the amplified sequences of the middle region of the 660 bp faithfully reproduced the sequences of the env gene of MMTV, an 18-mer sequence within the env gene was used as a probe for the 660 bp amplified sequence. The 18-mer probe was 5' end-labeled with ^{32}P -ATP using T4 polynucleotide kinase and purified by the NENSORB nucleic acid purification cartridge (NEN). Southern blot hybridization was performed using the conditions described by (Saiki et al., 1985, Science 230:1350-1354).

The product of the PCR (660 bp or 250 bp) was cloned directly from the reaction mixture into the TA cloning vector (Invitrogen) using the TA cloning kit and following the conditions recommended by the supplier. Direct cloning of the fragment isolated from the gel, was also performed. Plasmid DNA was purified by CsCl density gradient centrifugation or by precipitation with polyethylene glycol (Sambrook et al., 1989, in "Molecular Cloning/A Laboratory Manual", Cold Spring Harbor), restricted with HindIII and EcoRI, electrophoresed in 2% agarose gels and transferred to nitrocellulose filters. Southern blot hybridization was carried out using a 5'-terminal labeled internal probe as described above. Cloning procedures were performed in laboratories totally separate from those where PCR was carried out. Automated DNA sequencing (using Applied Technology Sequencer Model 373A) was performed in the Brookdale Molecular Biology Center. Sequence homology was determined using the IBI MacVector GenBank and GCG Programs.

To prevent contamination of the samples, processing of human tissues was performed in a laminar flow hood. DNA extractions were done in a chemical hood located in a different room from that where PCR was performed. PCR assays were assembled in a biological hood provided with ultraviolet light. Aerosol resistant tips and dedicated positive-displacement pipettes were used throughout. All equipment used for PCR (microcentrifuge, electrophoresis apparatus, pipettors) was cleaned each time with 10% sodium hypochlorite to assure DNA decontamination (Prince and Andrus, 1992, Biotechniques 12:358-36). After the initial experiments were performed, the plasmid containing the MMTV env gene was frozen and never used again, to avoid contamination. However, to detect plasmid contamination from our own env gene clones,

primers were designed to amplify plasmid sequences. All the authentic MMTV env positive samples were then tested and found negative for plasmid contamination.

Southern blotting and hybridization were performed as described (Southern, E.M., 1975, J. Mol. Biol. 98:503-517), using the 660 bp cloned sequences labeled by the random primer procedure (Feinberg, A.P., et al., 1983, Anal. Biochem. 132:6-13). Prehybridization and hybridization were performed in a solution containing 6 x SSPE, 5% Denhardt's, 0.5% SDS, 50% formamide, 100 µg/ml denaturated salmon testis DNA, incubated for 18 hrs at 42°C, followed by washings with 2 x SSC and 0.5% SDS at room temperature and at 37°C and finally in 0.1 x SSC with 0.5% SDS at 68°C for 30 min (Sambrook et al., 1989, in "Molecular Cloning/A Laboratory Manual", Cold Spring Harbor). For paraffin-embedded tissue sections the conditions described by Wright and Manos (1990, in "PCR Protocols", Innis et al., eds., Academic Press, pp. 153-158) were followed using primers designed to detect a 250 bp sequence.

6.2. Results

6.2.1. Selection of Specific MMTV Env Gene Sequences

A computer search for MMTV env gene homologous sequences was first performed, since sequence homology between the human endogenous retroviral sequences and MMTV had been described. The prototype of this group of human endogenous retroviruses is HERV-K10 (Ono, M. et al., 1986, J. Virol. 60:589-598). The sequences of the env gene of MMTV (Majors, I.E. and Varmus, H.E., 1983, J Virol 47:495-504) were aligned with sequences of the env gene of the human endogenous retrovirus HERV-K10 (Ono, M. et al., 1986, J. Virol. 60:589-598), using the IBI/Pustell Sequence Analysis Program. A region of 660 bp of low homology (16%) was localized between MMTV env gene sequences 976 and 1640 (Majors, I.E. and Varmus, H.E., 1983, J Virol 47:495-504). This

internal domain of the outer membrane of the env gene has only one glycosylation site and is highly conserved between strains. Two primers comprising 15 bp sequences at positions 976-990 (primer 1) and 1626-1640 (primer 3) were first synthesized. Later longer primers were synthesized (1N and 3N). An 18-mer sequence in the middle of the 660 bp MMTV env region (1388-1405) (primer 2) was used as a probe to identify the 660 bp sequence. A second oligomer probe was synthesized comprising the sequence 1554 to 1568 (primer 2a) to be used for hybridization when a sequence of around 250 bp (between positions 1388 and 1640) was amplified. For nested PCR reactions (Mullis, K.B. and Faloona, F.A., 1987, Meth Enzymol 155:335-350), another primer comprising sequences 1647 to 1661 (primer 4) was synthesized to be used with primer 1 in the first reaction and primers 2 and 3 in the second. Modified primers with GC clamps and extra sequences were also synthesized and used in the PCR (primers 1a and 3a). Another set of primers comprising sequences 974 to 1003 (5L) and 1558 to 1577 (3L) were subsequently developed because their T_m's matched and provided better amplification than the original primers. The sequences are represented in Table 1. All of them were productive in amplification reactions.

Table 1. Primer and probe sequences and location in mouse mammary tumor virus env gene

Designation	Sequence (5'-3')	Location
5		
1	CCTCACTGCCAGATC	976-990
1a	GGGAATTCCTCACTGCCAGATC	976-990
1N	CCTCACTGCCAGATCGCCT	976-993
2	TACATCTGCCTGTGTTAC	1388-1405
10	2N	CCTACATCTGCCTGTGTTAC
	2a	CCGCCATACGTGCTG
	3	ATCTGTGGCATAACCT
	3a	GGGAATTCATCTGTGGCATAACCT
	3N	ATCTGTGGCATAACCTAAAGG
15	4	GAATCGCTTGGCTCG
	5L	CCAGATCGCCTTTAAGAAGG
	3L	TACAGGTAGCAGCACGTATG

6.2.2. Detection of MMTV-Like Env Gene Sequences in Human Breast Tumor DNA

20 PCR was performed on DNA extracted from breast cancer tissues, normal breast tissues and from the plasmid containing the env gene of MMTV, using primers 1 and 3. Photographs of the ethidium bromide stained gels of the PCR product reveal the presence of an

25 approximately 660 bp sequence in some of the tumors, (Fig. 1A, lanes 1 and 3) but not in the normal tissue samples (Fig. 1A, lanes 2 and 4). As a positive control the MMTV env gene was also amplified (Fig. 1A, lane E). Similar results were obtained with modified

30 primers 1a, 3a, 3L and 5L. Southern blot hybridization of the gel with ³²P-labeled 18-mer oligonucleotide (primer 2) indicated that this internal sequence was present in the amplified material (Fig. 1B) and that the bands in the gel were not artifactual.

35 Our initial effort was to analyze a representative sample of breast cancer specimens as well as normal

tissues and other tumors. To date 343 breast tumors have been processed, DNA extracted and PCR preformed. Of these 343 tumors, 314 were carcinomas and 29 were fibroadenomas. Amplification of sequences of 660 bp was observed in 121 of the carcinomas (38.5%) and in 2 of the 29 fibroadenomas (6.9%). These sequences were confirmed to be MMTV env gene-like sequences by hybridization with the labeled specific probe containing the internal sequences. These sequences were not detected in the DNAs extracted from 20 normal organs, 23 cancers from other organs and 26 samples of blood lymphocytes including 7 from breast cancer patients whose breast specimens were positive. From 107 samples of normal breast obtained from reduction mammoplasties, 2 were positive (1.8%). In addition to DNA from lymphocytes from seven positive patients, DNA from their normal breast tissue of the operated breast was tested in 4 cases. All were negative (Table 2). Finally, DNA of the MCF-7, and ED (a cell line developed in our laboratory from the pleural effusion of a patient with an env -positive breast tumor) breast cancer cell lines were shown to contain the 660 bp MMTV env gene-like sequences (Table 3), while four other breast cancer cell lines were positive only for the 250 bp sequence (T47-D, BT-474, BT-20 and MDA-MB-231).

Table 2. Detection of MMTV env gene-like sequences in human DNA extracted from fresh or frozen tissues

5	<hr/>			
	Sample	Number	MMTV <u>env</u> gene sequences	% Positive
<hr/>				
	Breast Carcinomas	314	121	38.5%
	Breast Fibroadenomas	29	2	6.9%
10	Normal Breasts	107	2	1.8%
	*Normal Breasts	4	negative	
	Tumors other than breast	23	negative	
	Normal tissues	20	negative	
15	Lymphocytes	26	negative	
	**Lymphocytes	7	negative	
<hr/>				
	* Histologically normal tissue from same breast as positive cancer.			
20	** Lymphocytes from breast cancer patients who were positive for MMTV <u>env</u> gene sequences in the tumor.			

Tabl 3. Detection of MMTV env gene-like sequences in DNA from human cell lines in culture

	Human Cell Lines		MMTV <u>env</u> gene sequence
5	MC-7	(breast carcinoma)	positive
	T47-D	" "	negative
	BT-20	" "	negative
	MDA-MB-231	" "	negative
	ZR-75-1	" "	negative
10	SK-BR 3	" "	negative
	BT474	" "	negative
	ED	" "	positive
	MCF-10	(normal breast)	negative
	HB-447	" "	negative
15	HL-60	(promyelocytic leukemia)	negative
	K562	(erythroleukemia)	negative
	Jurkat	(T cell leukemia)	negative
	Hep 6-2	(hepatoma)	negative

The nested polymerase reaction was used in several instances to increase sensitivity and specificity, thus reducing the probability of false positives. In Fig. 2, results of a representative nested reaction are shown using primers 1 and 4 in the first reaction (Fig. 2A) and 2 and 3 for the 2nd reaction. The specificity of the reaction can be seen in the 2nd amplification (Fig. 2B).

To study a large number of samples and to be able to perform archival studies, PCR of paraffin-embedded tissue sections was also carried out. Primers 2 and 3 were used to amplify a 250 bp sequence within the 660 bp stretch when DNA was extracted from paraffin-embedded tissue sections since larger size sequences are difficult to amplify after fixation. Tumor DNA was amplified (Fig. 3A, lanes 2-5) whereas normal breast DNA was not (Fig. 3A, lane 1). The identification of

this 250 bp sequence with the MMTV-like env gene was confirmed by hybridization with an internal probe (primer 2a) as shown in Fig. 3B. Using this procedure we have analyzed 151 breast cancer samples and found that 60 (39.7%) possess the 250 bp sequence. Of the 27 normal breast samples obtained from reduction mammoplasties assayed by this procedure, one was positive (3.7%). These results, in conjunction with those obtained from lymphocytes and from normal breast tissue of patients whose breast cancer was PCR positive, indicate that MMTV-like sequences are present in a significant number of human breast cancer DNA which cannot be explained by DNA polymorphism.

6.2.3. Cloning and Sequencing of the MMTV-Like Env Gene Sequences

To find out whether there was homology to MMTV env gene throughout the whole 660 bp stretch, the product of the PCR from 8 different tumors was cloned and sequenced. In Fig. 4 the sequence of different clones comprising around 600 bp are represented, as aligned to the MMTV env gene sequence of the GR and BR6 strains (Redmon, S. and Dickson, C., 1983, EMBO J. 2:125-131). This domain of the env gene in the GR strain is 100% homologous to the C₃H strain and 98% to the BR6 strain (Majors, I.E. and Varmus, H.E., 1983, J. Virol. 47:495-504; Moore, R. et al., 1987, J. Virol. 61:480-490). Evaluation of the clones indicated that homology to MMTV env gene varied from 95% to 99%. Another seven clones comprising only 250 bp were also sequenced. Homology to MMTV env gene varied from 95% to 99% (data not shown). When compared to the human endogenous provirus HERV-K10, the homology of all the clones was less than 15%. When compared against all known viral and human genes (more than 130,000 entries) using the 1B1 MacVector GenBank and GCG programs, the highest homology recorded was 18%.

6.2.4. Southern Blot Analysis Using Cloned Sequences

To investigate whether the env gene-like sequences were present in human DNA, Southern blot hybridization was performed using the cloned sequence as probe. DNAs from normal breast tissues, env positive or negative breast tumors, tumors other than breast and breast cancer cell lines were restricted with EcoRI and in some instances with PstI, BglII or KpnI. EcoRI is a frequent cutter restriction enzyme that digests MMTV proviral DNA between env and pol genes. Four different cloned 660 bp sequences were used as probes after labeling with ³²P by random prime-labeling. Results of some of the Southern blot hybridization experiments are shown in Fig. 5. They reveal the presence of a labeled restriction fragment migrating at approximately 7-8 kb in breast cancer DNA, in ED and two fragments in MCF-7 cells. Different restriction patterns were observed with the other three enzymes. The 660 bp sequence was absent in 10 normal tissues, 10 fibroadenomas and 10 tumors from other tissues. It is important to emphasize that hybridization conditions for these experiments were stringent (as described in Section 6.1) to avoid interference with endogenous sequences that might interact with the probes.

7. Example: Detection of a Retrovirus Proviral Fragment in Human Breast Cancer Cells and Tissues

7.1. Materials and Methods

To detect longer retrovirus proviral fragments in breast cancer samples, DNA was extracted from breast cancer carcinoma tissue samples as described above in Section 6.1. Two rounds of long PCR was performed on the DNA primers 5L (SEQ ID NO:11) and LTR3 (SEQ ID NO:19). The primer 5L contains nucleotides 7370-7395 of the MMTV BR6 genome (5'-3': CCAGATCGCCTTTAAGAAGG) (SEQ ID NO:11) and primer LTR3 contains nucleotides

9918-9927 of the MMTV BR6 genome (5'-3':
CGAACAGACACAAAGCGACG) (SEQ ID NO:19). Long PCR was
performed using protocols described by the manufacturer
(Perkin Elmer, Foster City, CA). The amplified
5 retroviral fragment isolated from the breast cancer
sample was cloned into the TA cloning vector
(Invitrogen) and automated sequencing was performed
as described in Section 6.1.

7.2 Results

10 An approximately 2.6 kb retroviral fragment
containing 1,228 bp of the 3' LTR sequence and 1,336 bp
of the env gene sequence of a potential provirus was
detected in a human breast carcinoma tissue sample by
the long PCR technique using the 5L and LTR3 primers.
15 The sequence of this retroviral fragment is shown in
Fig. 9. (SEQ ID NO:20).

When compared with the two strains of MMTV C3H
and BR6, the sequence homology was 90.8% and 90.7%,
respectively, over the MMTV genomic fragment from
20 nucleotides 7370-9937. When compared with the
endogenous retroviral sequences (HUMERKA), sequence
homology was only 58% in 36 bp and 71% in 74 bp.

8. Discussion

Search for virus-related sequences in human breast
25 cancer has been hampered by great variation reported
in previous studies, by the presence of endogenous
retroviral sequences in human DNA and by the lack of
sensitivity of the methods employed. The studies
reported herein circumvent these deficiencies by
30 focusing on sequences with low homology to human
endogenous retroviruses, by investigating a large
number of tumors and several types of controls and
by using the most sensitive technology presently
available.

35 The results indicate that unique MMTV env gene
sequences were present in 38.5% of the breast cancer

samples analyzed and 39.7% of archival samples of breast cancer and that these sequences were absent in normal tissues including lymphocytes from patients with positive breast cancer and in cancers other than breast. Normal breast tissue and fibroadenomas had a low frequency (1.8 to 6.9%) of positive results. When cloned and sequenced, the sequences were found to be highly homologous to MMTV env gene, but not to the endogenous retroviral sequences. Furthermore, experiments in which the cloned amplified sequences were used for hybridization with DNA from breast cancer or normal tissues revealed that homologous DNA was only present in breast cancer DNA. The results also indicate that a human breast carcinoma sample contained an about 2.6 kb MMTV-like fragment comprised of 1,336 bp of the env gene and 1,228 bp of the 3' LTR.

The detection of MMTV env gene sequences in two fibroadenomas out of 29 and in two normal breast tissue samples out of 107 samples is of uncertain significance. Although such results could potentially be artifactual, and thus may represent false positives, they may alternatively indicate the presence of histologically unrecognized cells that were or will be neoplastic.

Ninety percent (90%) of the breast cancers tested were invasive ductal carcinomas, which reflects the prevalence of this type of neoplasm. Most patients were node-positive which is probably artifactual since it was necessary that tumor size be sufficiently large to provide an aliquot for research and tumor size correlates with node positivity.

It is unlikely that differences in homology between MMTV env gene and the cloned human sequences are generated by errors committed by the Taq polymerase. It has been estimated that the rate of nucleotide misincorporation is 1×10^{-5} per cycle (Ehrlich et al, 1991, Science 252:1643-1651) and

therefore, only a total of 0.32 nucleotides
misincorporated should be expected in 660 bp after
50 cycles. The differences in homology between clones
from different patients is likely to represent
5 heterogeneity of the env gene.

In contrast to earlier, ambiguous data associating
MMTV-like sequences with human breast cancer, we have
clearly demonstrated the existence of such sequences in
breast cancer cells which cannot be explained by any
10 known human endogenous retroviral sequence. Our data do
not support the results of earlier studies which
indicated that, as in the mouse, MMTV-like sequences
were found in lymphocytes from two patients with breast
cancer (Crepin, M. et al., 1984, Biochem. Biophys. Res.
15 Comm. 118:324-331). The absence of MMTV env-like
sequences in lymphocytes could reflect the fate of a
unique lymphocyte subset over decades between initial
encounter and the appearance of clinical breast cancer;
alternatively, the human disease may differ from the
20 mouse model. Results from attempts to identify unique
MMTV-like pol gene sequences have shown that they
cannot be distinguished from the reverse transcriptase
sequences of endogenous retroviruses (Deen, K.C. and
Sweet, R.W., 1986, J. Virol. 57:422-432).

25 The origin of the MMTV env gene-like and 3' LTR-
like sequences found in tumor DNA could be the result
of integrated MMTV-like sequences from a human mammary
tumor virus. Polymorphism of endogenous retroviral
sequences is conceivable but can be ruled out because
30 these sequences were not detected in lymphocytes from
the positive patients, in sections of the cancerous
breast from which abnormal cells were absent, or in
normal breast tissue from patients with MMTV env-like
positive tumors. Recombination during tumorigenesis
35 between endogenous sequences to resemble the MMTV env
genes seems highly unlikely since no known gene or
viral sequence is more than 18% homologous to the

660 bp sequence. The longer about 2.6 kb MMTV-like fragment detected in a human breast carcinoma had minimal homology (58% in 36 bp and 71% in 74 bp) to endogenous human retroviral sequences. Thus, the most conservative interpretation is that our findings represent exogenous sequences from an agent similar to MMTV. Recombination between endogenous and exogenous env gene sequences are known to accelerate the development of malignancies in mice (DiFronzo, N.L. and Holland, C.A., 1993, J. Virol. 67:3763-3770). Whether the MMTV-like sequences belong to an entire acquired provirus or to an exogenous fragment integrated into endogenous sequences, is presently not known. Experiments are in progress to distinguish between these possibilities.

Several genetic alterations have been identified in human breast cancer that can be useful as markers for prevention, detection or prognosis (reviewed in Runnenbaum, I. et al., 1991, Proc. Natl. Acad. Sci. USA 88:10657-10661). The BRCA1 and BRCA2 genes have recently been described. They account for at least 5% of breast cancer and are related to familial breast cancer (Miki, Y. et al., 1994, Science 266:66-71; Wooster, R. et al., 1994, Science 265:2088-2090). We have primary evidence that familial clustering of the MMTV env gene-like sequences occurs, accounting for an even higher percentage of cancers in affected families (Holland et al. 1994, Proc. Am. Assoc. Cancer Res 35:218). The presence of MMTV-like sequences may be correlated with special clinical disease status, may provide another potential molecular marker, and may distinguish a subset of human breast cancer for which viral etiology is tenable. This has implications for epidemiology, therapy and prevention.

Various publications are cited herein, the contents of which are hereby incorporated by reference in their entireties.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: HOLLAND, JAMES

5 (ii) TITLE OF THE INVENTION: DETECTION OF MAMMARY TUMOR VIRUS-LIKE
SEQUENCES IN HUMAN BREAST CANCER

(iii) NUMBER OF SEQUENCES: 20

(iv) CORRESPONDENCE ADDRESS:

10 (A) ADDRESSEE: Brumbaugh, Graves, Donohue & Raymond
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(C) CITY: New York
(D) STATE: NY
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(F) ZIP: 10112-0228

(v) COMPUTER READABLE FORM:

15 (A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:

20 (A) APPLICATION NUMBER: NOT YET ASSIGNED
(B) FILING DATE: 08-NOV-1996
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

25 (A) APPLICATION NUMBER 08/555,394
(B) FILING DATE: 09-NOV-1995

(viii) ATTORNEY/AGENT INFORMATION:

30 (A) NAME: Kole, Lisa B
(B) REGISTRATION NUMBER: 35,225
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(A) TELEPHONE: 212-408-2628
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35 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

45 (vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCTCACTGCC AGATC

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGGAATTCCT CACTGCCAGA TC

22

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCTCACTGCC AGATCGCCT

19

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TACATCTGCC TGTGTTAC

18

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
CCTACATCTG CCTGTGTTAC

20

(2) INFORMATION FOR SEQ ID NO:6:
10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
15 (ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
20 (vi) ORIGINAL SOURCE:
(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
CCGCCATACG TGCTG

15

(2) INFORMATION FOR SEQ ID NO:7:
25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
30 (ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:
35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
ATCTGTGGCA TACCT

15

(2) INFORMATION FOR SEQ ID NO:8:
40 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
45 (ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGGAATTCAT CTGTGGCATA CCT

23

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATCTGTGGCA TACCTAAAGG

20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GAATCGCTTG GCTCG

15

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCAGATCGCC TTTAAGAAGG

20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TACAGGTAGC AGCACGTATG

20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Leu Lys Arg Pro Gly Phe Gln Glu His Glu Met Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Gly Leu Pro His Leu Ile Asp Ile Glu Lys Arg Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: N-terminal
5 (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Thr Asn Cys Leu Asp Ser Ser Ala Tyr Asp Thr Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:16:

- 10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- 15 (ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Asp Ile Gly Asp Glu Pro Trp Phe Asp Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:17:

- 25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 662 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- 30 (ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

35	TCCTCACTGC	CAGATCGCCT	TTAAGAAGGA	CGCCTTCTGG	GAGGGAGACG	AGTCTGCTCC	60
	TCCACGGTGG	TTGCCTTGCG	CCTTCCCTGA	CCAAGGGGTG	AGTTTTTCTC	CAAAAGGGGC	120
	CCTTGGGTTA	CTTTGGGATT	TCTCCCTTCC	CTCGCCTAGT	GTAGATCAGT	CAGATCAGAT	180
	TAAAAGCAAA	AAGGATCTAT	TTGGAAATTA	TACTCCCCCA	GTCAATAAAG	AGGTTTCATCG	240
	ATGGTATGAA	GCAGGATGGG	TAGAACCCTAC	ATGGTTCTGG	GAAAATTCTC	CTAAGGATCC	300
40	CAATGATAGA	GATTTTACTG	CTCTAGTTCC	CATACAGAAT	TGTTTCGCTT	AGTTGCAGCC	360
	TCAAGATATC	TTATTCTCAA	AAGGCAGGAT	TTCAGGAACA	TGAGATGATT	CCTACATCTC	420
	TGTGTTACTT	ACCCTTATGT	CATATTATTA	GGATTACCTC	AGCTAATAGA	TATAGAGAAA	480
	GAGGATCTAC	TTTTCATATT	TCCTGTTCTT	CTTGTAGATT	GACTAATTGT	TTAGATTCTT	540
	CTGCCTACGA	CTATGCAGCG	ATCATAGTCA	AGAGGCCGCC	ATACGTGCTG	CTACCTGTAG	600
45	ATATTGGTGA	TGAACCATGG	TTTGATGATT	CTGCCATTCA	AACCTTTAGG	TATGCCACAG	660
	AT						662

(2) INFORMATION FOR SEQ ID NO:18:

- 50 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 663 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

5	TCCTCACTGN	CAGATCGCCT	TTAAGAAGGA	CGCCTTCTGG	GAGGGAGACG	AGTCTGCTCC	60
10	TCCACGGTGG	TTGACTTGCG	CCTTCCCTGA	CCAGGGGGTG	AGTTTTTCTC	CAAAAGGGGC	120
	CCTTGGGGTTA	CTTTGGGATT	TCTCCCTTCC	CTCGCCTAGT	GATAGTCAGT	CAGATCAGAT	180
	TAAAAGCAAA	AAGGATCTAT	TTGGAAATTA	TACTCCCCCT	GTCAATAAAG	AGGTTCATCG	240
	ATGGTATGAA	GCAGGATGGG	TAGAACCCTAC	ATGGTTCTGG	GAAAATTCTC	CTAAGGATCC	300
	CAATGATAGA	GATTTTACTG	CTCTAGTTCC	CATACAGAAT	TGTTTCGCTT	AGTTGCAGCC	360
15	TCAAGATATC	TTATTACAA	AAGGCAGGAT	TTCAAGAACA	TGACATGAAT	CCCTACATCT	420
	CTGTGTTACT	TACCCTTATG	CCANANTATT	AGGATTACCT	CAGCTAATAG	ATATAGAGGA	480
	AGAGGATCTA	CTTTTCATAT	TTCTGTCTCT	TCTTGTAGAT	TGACTAATTG	TTTAGATTCT	540
	TCTGCCTACG	ACTATGCAGC	GATCATAGTC	AAGAGGCCGC	CATACGTGCT	GCTACCTGTA	600
20	GATATTGGTG	ATGAACCATG	GTTTGATGAN	NCTGCCANTC	AAACCTTTAG	GTATNCCACA	660
	GAT						663

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGAACAGACA CAAACACACG

20

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2598 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

	CGAACAGACA	CAAACACACG	AGAGGTGAAT	GTTAGGACTG	TTGCAAGTTT	ACTCAAAAAA	60
	CAGCACTCTT	TTATATCATG	GTTTACATAA	GCATTTACAT	AAGACTTGGA	TAAGTTCCAA	120
	AAGAACATAG	GAGAATAGAA	CACTCAGAGC	TTAGATCAAA	ACATTTGATA	CCAAACCAAG	180
5	TCAGGAAACC	ACTTGTCTCA	CATCCTTGTT	TTAAGAACAG	TTTGTGACCC	TGAACTTACT	240
	TAAACCTTGG	GAACCGCAAN	GTTGGGCTCA	TAAAGGTTAT	CCATTATAGC	TCATGCCAAA	300
	ATTATCTGCA	GAAATGTGTT	CCTAATTGTC	TAGCCACTGC	CCCCTCCCTT	GGTATAATGA	360
	AAATCTTTCC	CCCAACGTTT	ATCCCACTCC	CCTAGATAAA	TATAATCATG	TACCTGTTGT	420
	TTTATGTTCG	CTTTTTCTTC	CTGAGTTAAC	ACACACCAAG	GAGGTCTAGC	TCTGGCGAGT	480
10	CTTTCACGAA	AGGGGAGGGA	TCTGTACAAC	ACTTTATAGC	CGTTGACTGT	GACCCACCTA	540
	TCGAAATTTA	AATCGTATCT	TCCTGTATAT	GGTAGCGGGG	CGTCTGTTGG	TCTGTAGATG	600
	TAAGTCCCGG	TTGCCACCAC	CTGTCTCCTA	TTTTGACAAG	CGTACTCCTC	TTTCCCCTTT	660
	TTACTTCTAG	GCCTGAGGCC	CTTAGTCCTT	GCACCTGTTT	TTCAACTGAG	GTTGAGCGTC	720
	TCCTTCTATT	TTCTATTCCC	ATTTCTAACC	TTTGAATTTG	AGTAAATATA	GTGCTAAAAG	780
15	ACAAAGATTG	ATTTCTTAAC	ATCATGATTA	ATAATCGACC	TATTGGATTG	GTCTTATTGG	840
	TAAAAATATA	ATTTTCTAGC	AGCATTCTTA	TTTCTATTTT	TGAAGGACAA	AGTCGGTGTG	900
	GCTTGTANA	GGAANTTGGC	TGTGGTCCTT	GCCCCACGAG	GAAGGTCGAG	TTCTCCGAAT	960
	TGTTTAGATT	GTAATCTTGC	ACAGAAGAGT	TATTTAAAAGA	ATCAAGGGTG	AGAGCCCTGC	1020
	GAGCACGAAC	CGCAACTTCC	CCCAATAGCC	CCAGGCAAAG	CAGAGCTATG	CCAAGTTTGC	1080
20	AGCAGANAAT	GAGTATGTCT	TTGTCTGATG	GGCTCATCCG	CGTGACGCA	GACGGGTCGT	1140
	CCTTGGTGGG	AAACAACCCC	TTGGCTGCTT	CTCTCCTAAG	TGTAGGACAC	TCTCGGGAGT	1200
	TCAACCATTG	CTGCTGCAGG	CGCGGCATTT	CCCCCTTTTT	TCTTTTTTAA	AAGAAGCACG	1260
	TTAAGATCTG	ACTGCACTTG	GTCAAGGCTC	TTCGCAAAGC	ACTGGAAAAT	AACGGGGAAA	1320
	ATCATAAGTA	CTATGACCAA	AAGCAGGGCT	CCAACTCCTA	TAAAAATGAA	ATATTGTGTT	1380
25	CTAATCCAAT	GGATTTAAAG	CCTTTACTCC	ATTGGCNAAG	GANTGANCCA	ACCCCTGAGG	1440
	TCCCTGCGTT	CAAATTTTTT	TGCTCNTATC	CTAATCCAAT	TGGTAACCCC	GTTTNTTTTT	1500
	GAAACTCATG	TCTTCAAATG	CCCAATAAAT	GAGCCCTGGT	TCTTTCCCAG	CTCTCAGAAG	1560
	CATTATACGG	NANAGGTGTG	ACACAGCATA	AAATCATAAT	TTGCATGACA	CCTAGTGGAC	1620
	ATTCTGGTCT	TTAAGTTTGC	CACATCTTGT	CCCAACTCTA	AAACTACTTC	TTCTAAAGCA	1680
30	TTAAGTCTAG	CTTTCAATTT	TAAGTCTATT	ATTCTTTGTT	CAGATNAGGC	TAATGTAACA	1740
	TTTCTATGAA	GATTATTAAC	AAACGTAGCA	GTTTGCATCT	CCTTAACTAA	GGCAGTAGTA	1800
	GCTACAGCAA	AGGAAGTGAT	AATAGCAATT	AAAGCAGATA	TGCCCAGAAT	AATGGCAGCG	1860
	ACGAATCGCT	TAGCTCGAAT	TAAATCTGTG	GCATACCTAA	AGGTTTGAAT	GGCAGAATCA	1920
	TCAAACCATG	GTTTCATCACC	AATATCTACA	GGTTACAACA	CATATGGCGG	CCCCTTGAAT	1980
35	ATGAATCGCT	GCATATCCGT	NGGCAAAAAA	TCTAACCATT	ATTCTCTCTN	CCNAAAAACG	2040
	GGATTTGAAA	NTTATNCCCC	TTNCCCCNAA	CCCANACCGA	GGTACCCCAT	AATGNNGGGG	2100
	GTATCTANAA	NAGGGCATAG	GGGTAAGAAA	AACGGCAGAG	NGGGATCNTT	TATGTTTCNGG	2160
	AAATTCNNGG	TTTGGGAGAA	TAAGATTCTG	GAGGCTGCAA	ATTAAGGGAA	ACATTNTGTA	2220
	TGGGGAATAG	AGCAGTAAAA	TCTCTATCAT	GGGGATCTTT	AGGGAGAATT	TTCCCAGGAA	2280
40	CCAAGTAGGT	TCNAACCCAT	CNTGCTTCAT	ACCATCGATG	AACNTCTTTA	TTGACAGGGG	2340
	GAGTATAATT	TCCAAATAGA	TCCTTTTTGT	TTTTAATCTG	ATCTGACTGA	TCTACACTAG	2400
	GCGGGGGAAG	GGAGAAATCC	CAAAGTAACC	CAAGGGCCCC	TTTTGGAGAA	AAACTCACCC	2460
	CCTGGTCAGG	GAAGGCGCAA	GGCAACCACC	GTGGAGGAGC	AGACTCGTCT	CCCTCCCAGA	2520
	AGGCGTCCTT	CTTAAAGGCG	ATCTGGAGGA	GCAGACTCGT	CTCCCTCCCA	GAAGGCGTCC	2580
45	TTCTTAAAGG	CGATCTGG					2598

Claims

- 1 1. A composition comprising an oligonucleotide primer
2 which may be used to detect the presence of a
3 nucleic acid molecule which (i) hybridizes to the
4 env gene of a mouse mammary tumor virus; (ii) is
5 present in at least 38 percent of DNA samples
6 prepared from breast cancer tissue of different
7 human subjects; and (iii) hybridizes to less than
8 7 percent of DNA samples prepared from tissues
9 other than breast cancer tissue from different
10 human subjects.
- 1 2. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 CCTCACTGCCAGATC (SEQ ID NO:1).
- 1 3. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 GGGAATTCCTCACTGCCAGATC (SEQ ID NO:2).
- 1 4. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 CCTCACTGCCAGATCGCCT (SEQ ID NO:3).
- 1 5. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 TACATCTGCCTGTGTTAC (SEQ ID NO:4).
- 1 6. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 CCTACATCTGCCTGTGTTAC (SEQ ID NO:5).
- 1 7. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 CCGCCATACGTGCTG (SEQ ID NO:6).

- 1 8. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 ATCTGTGGCATACT (SEQ ID NO:7).
- 1 9. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 GGGAATTCATCTGTGGCATACT (SEQ ID NO:8).
- 1 10. The composition of claim 1, wherein the
2 oligonucleotide primer comprises a sequence
3 selected from the group consisting of
4 ATCTGTGGCATACTAAAGG (SEQ ID NO:9);
5 GAATCGCTTGGCTCG (SEQ ID NO:10);
6 CCAGATCGCCTTTAAGAAGG (SEQ ID NO:11); and
7 TACAGGTAGCAGCACGTATG (SEQ ID NO:12).
- 1 11. An essentially purified peptide encoded by a
2 nucleic acid molecule which (i) hybridizes to
3 a gene of MMTV; (ii) is present in at least
4 20 percent of DNA samples prepared from breast
5 cancer tissue of different human subjects; and
6 (iii) is present in less than 5 percent of DNA
7 samples prepared from tissues other than breast
8 cancer tissue from different human subjects.
- 1 12. An antibody which specifically binds to the
2 peptide of claim 11.
- 1 13. The peptide according to claim 11 which comprises
2 the amino acid sequence LKRPGFQHEMI (SEQ ID
3 NO:13).
- 1 14. An antibody which specifically binds to the
2 peptide of claim 13.
- 1 15. The peptide according to claim 11 which comprises
2 the amino acid sequence GLPHLIDIEKRG (SEQ ID NO:14).

- 1 16. A method of diagnosing breast cancer in a human
2 subject, comprising detecting the presence of a
3 peptide encoded by a nucleic acid molecule which
4 (i) hybridizes to the env gene of 3' LTR of a
5 mouse mammary tumor virus; (ii) is present in at
6 least 20 percent of DNA samples prepared from
7 breast cancer tissue of different human subjects;
8 and (iii) is present in less than 5 percent of DNA
9 samples prepared from tissues other than breast
10 cancer tissue from different human subjects.
- 1 17. The method according to claim 16, wherein the
2 presence of a peptide comprising the amino acid
3 sequence LKRPGFQHEMI (SEQ ID NO:13) is detected
4 by the binding of an antibody specific to the
5 peptide.
- 1 18. The method according to claim 16, wherein the
2 presence of a peptide comprising the amino acid
3 sequence GLPHLIDIEKRG (SEQ ID NO:14) is detected
4 by the binding of an antibody specific to the
5 peptide.
- 1 19. The method according to claim 16, wherein the
2 presence of a peptide comprising the amino acid
3 sequence TNCLDSSAYDTA (SEQ ID NO:15) is detected
4 by the binding of an antibody specific to the
5 peptide.
- 1 20. The method according to claim 16, wherein the
2 presence of a peptide comprising the amino acid
3 sequence DIGDEPWFDD (SEQ ID NO:16) is detected by
4 the binding of an antibody specific to the
5 peptide.
- 1 21. A composition comprising an oligonucleotide primer
2 which may be used to detect the presence of a

3 nucleic acid molecule which (i) hybridizes to a
4 nucleic acid comprised of a sequence selected from
5 the group consisting of the env gene and the 3'
6 LTR of a mouse mammary tumor virus; (ii) is
7 present in a substantial percentage of DNA samples
8 prepared from breast cancer tissue of different
9 human subjects; and (iii) hybridizes to less than
10 5 percent of DNA samples prepared from tissues
11 other than breast cancer tissue from different
12 human subjects.

1 22. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 CCAGATCGCCTTTAAGAAGG (SEQ ID NO:11).

1 23. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 CGAACAGACACAAACACACG (SEQ ID NO:19).

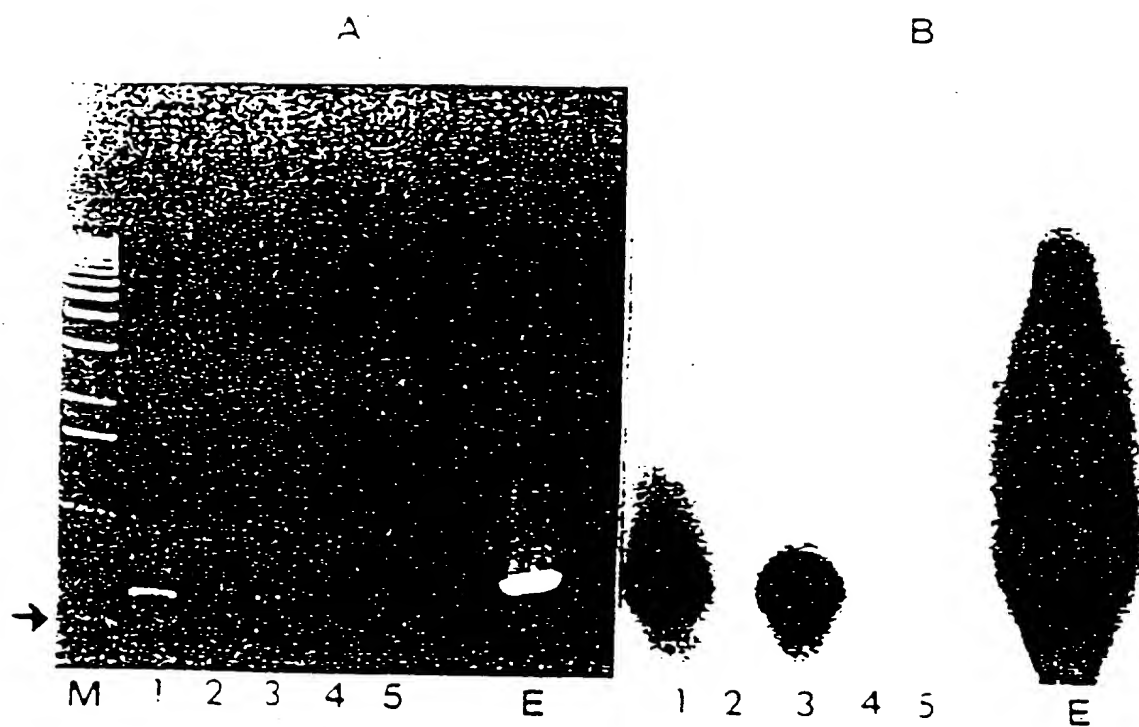


FIGURE 1

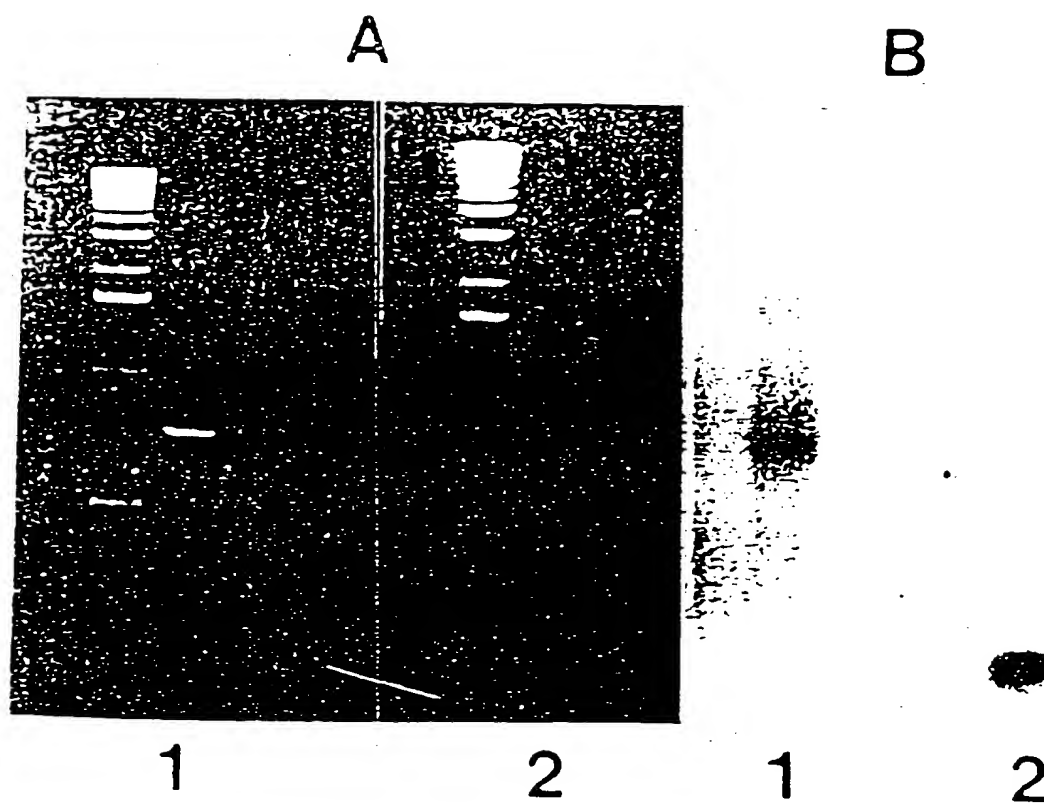


FIGURE 2

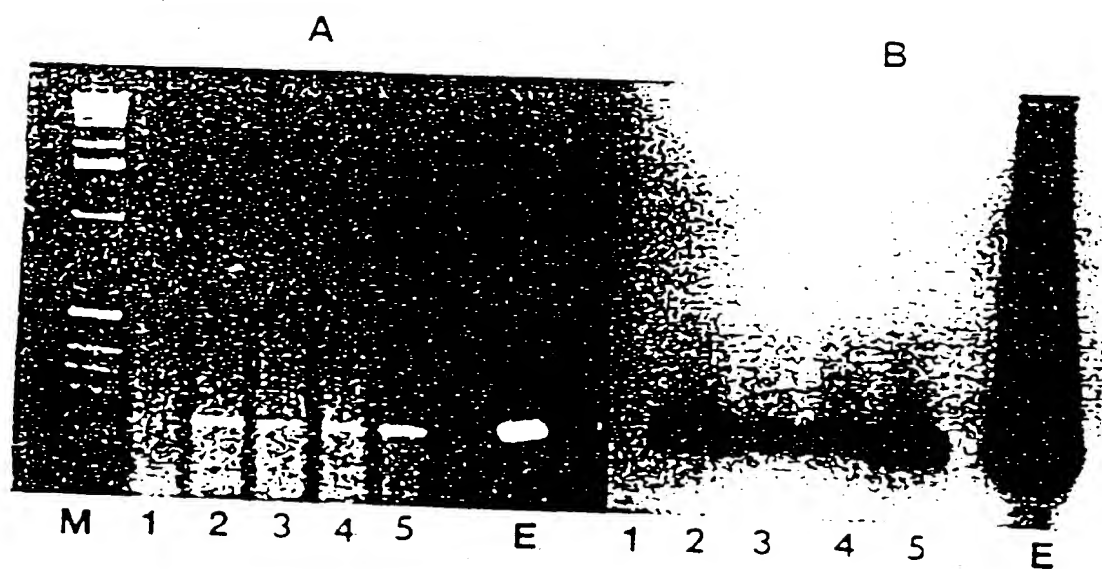


FIGURE 3

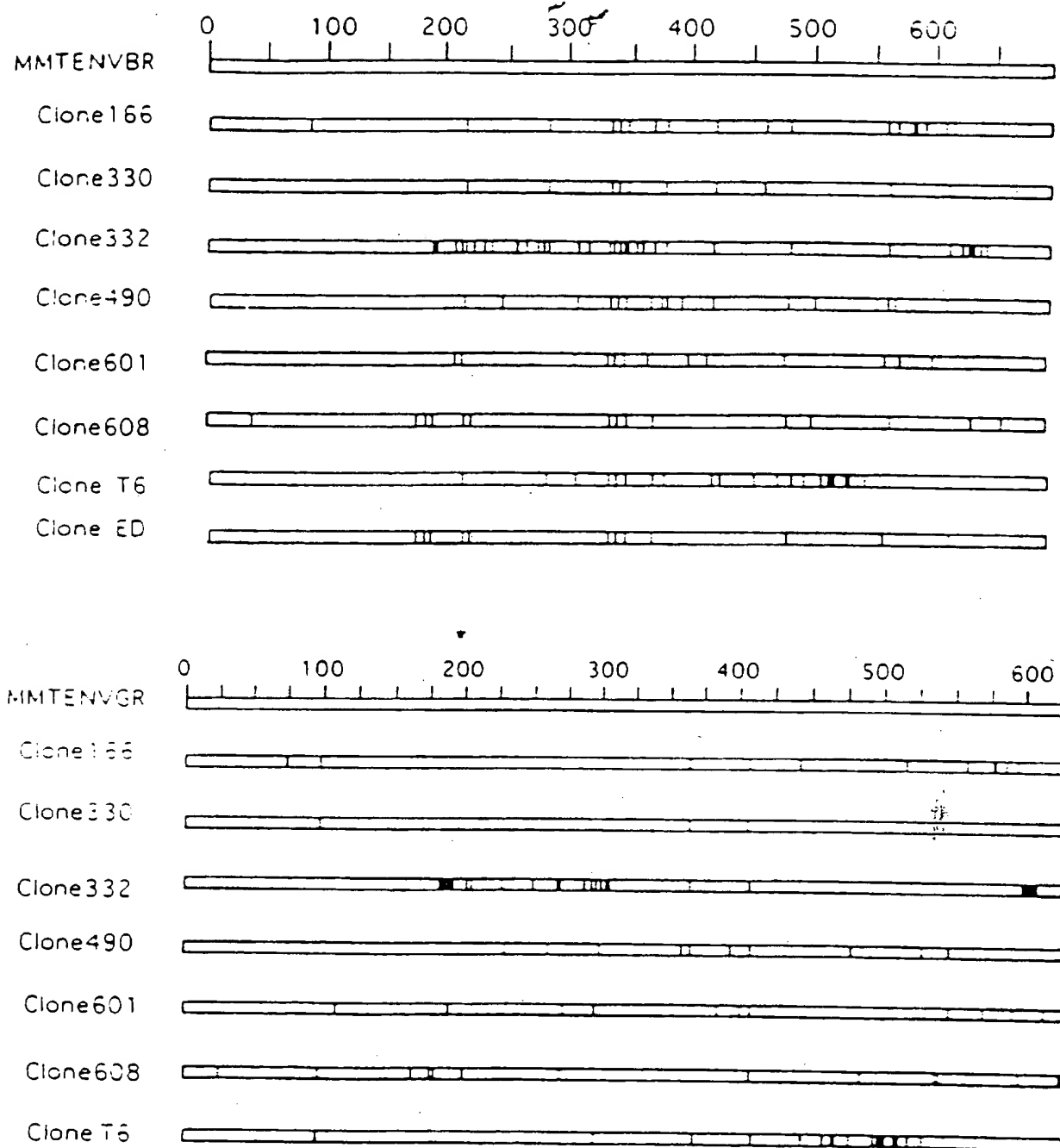


FIGURE 4

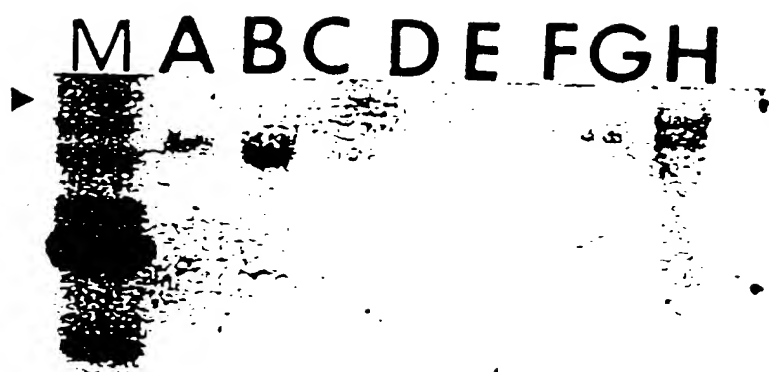


FIGURE 5

A B C D E F G H



FIGURE 6

6

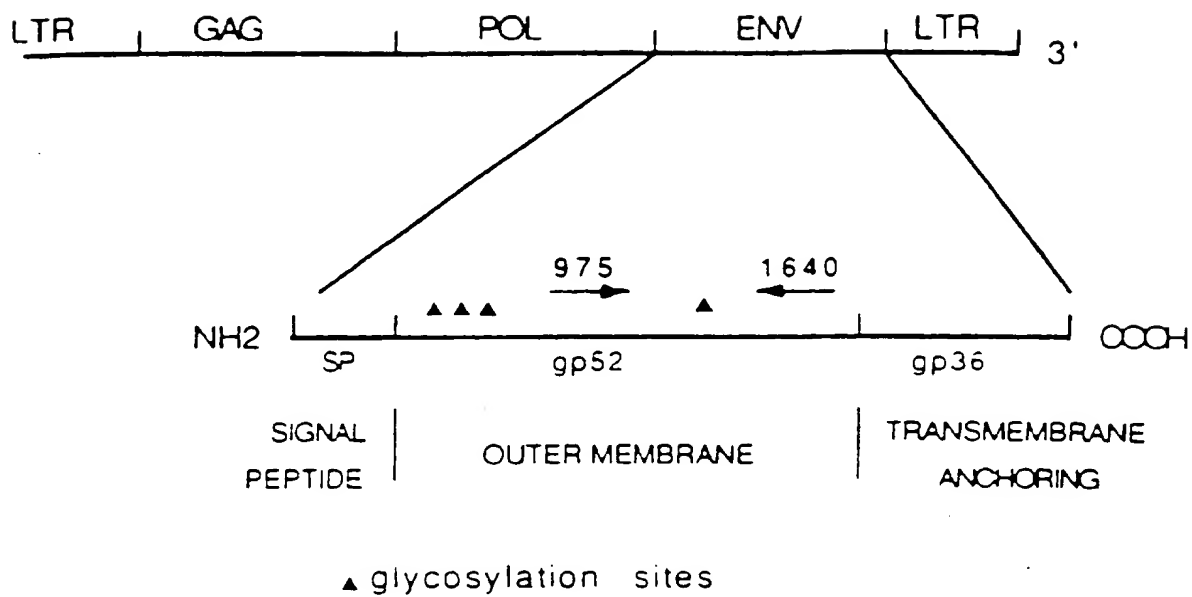


FIGURE 7

NTTENV 980 | 1000 | 1020 |
[1810] TCTCACTGGGAGATGGCT TTAGAAGGAGGCTTCTGG GAGGAGAGGCTCTCTCC
MS1627.Seq TCTCACTGGGAGATGGCT TTAGAAGGAGGCTTCTGG GAGGAGAGGCTCTCTCC
5 40 15 20 25 30 35 40 45 50 55 60

NTTENV 1040 | 1060 | 1080 |
[1810] TCCAGCGTGGTTGGCTTGG CCTTCCTGACCAAGGGCTG ACTTTTCTCCAAAAGGGC
MS1627.Seq TCCAGCGTGGTTGGCTTGG CCTTCCTGACCAAGGGCTG ACTTTTCTCCAAAAGGGC
65 70 75 80 85 90 95 100 105 110 115 120

NTTENV 1100 | 1120 | 1140 |
[1810] CCTTGGGTTACTTTGGGATT TCTCCCTTCCCTGGCTAGT GTAGATCACTCAGATCAGAT
MS1627.Seq CCTTGGGTTACTTTGGGATT TCTCCCTTCCCTGGCTAGT GTAGATCACTCAGATCAGAT
125 130 135 140 145 150 155 160 165 170 175 180

NTTENV 1160 | 1180 | 1200 |
[1810] TAAAGCCAAAAGGATCTAT TTGGAATTATACTCCCGCA GTCAATAAAGAGGTTTCATCG
MS1627.Seq TAAAGCCAAAAGGATCTAT TTGGAATTATACTCCCGCT GTCAATAAAGAGGTTTCATCG
185 190 195 200 205 210 215 220 225 230 235 240

NTTENV 1220 | 1240 | 1260 |
[1810] ATGGTATGAAGCAGGATGGG TAGAAGCTACATGGTTCTGG CAATAATTCTCTAAGGATCC
MS1627.Seq ATGGTATGAAGCAGGATGGG TAGAAGCTACATGGTTCTGG CAATAATTCTCTAAGGATCC
245 250 255 260 265 270 275 280 285 290 295 300

NTTENV 1280 | 1300 | 1320 |
[1810] CAATGATAGAGATTACTG CTCTAGTTCCCATACAGAT TCTTCTCTTACTTCCAGCC
MS1627.Seq CAATGATAGAGATTACTG CTCTAGTTCCCATACAGAT TCTTCTCTTACTTCCAGCC
305 310 315 320 325 330 335 340 345 350 355 360

NTTENV 1340 | 1360 | 1380 |
[1810] TCAAGATATCTTATTCCAA AAGCCAGGATTTCAAGACA TCAGATC-ATCTCTACATCT
MS1627.Seq TCAAGATATCTTATTCCAA AAGCCAGGATTTCAAGACA TCAGATC-ATCTCTACATCT
365 370 375 380 385 390 395 400 405 410 415 420

NTTENV 1400 | 1420 | 1440 |
[1810] CTGTGTACTTACGCTTATC CCACACTTATAGGATTAAGT CAGCTATATAGATATAGAA
MS1627.Seq CTGTGTACTTACGCTTATC CCACACTTATAGGATTAAGT CAGCTATATAGATATAGAA
425 430 435 440 445 450 455 460 465 470 475 480

NTTENV 1460 | 1480 | 1500 |
[1810] AGAGGATCTACTTTTCAAT TTCTGTCTTCTTTTATAGT TCACTAATTGTTTATGATTCT
MS1627.Seq AGAGGATCTACTTTTCAAT TTCTGTCTTCTTTTATAGT TCACTAATTGTTTATGATTCT
485 490 495 500 505 510 515 520 525 530 535 540

NTTENV 1520 | 1540 | 1560 |
[1810] TCTGCTTACGACTATCCAGC CATCACTACTCAGAGGCGGC CATACCTGCTCTACTCTATA
MS1627.Seq TCTGCTTACGACTATCCAGC CATCACTACTCAGAGGCGGC CATACCTGCTCTACTCTATA
545 550 555 560 565 570 575 580 585 590 595 600

NTTENV 1580 | 1600 | 1620 | 1640 |
[1810] GAGATTTGATGAGGAGATG GTTGTGATGAGCTTCCCACTC AAAGCTTTAGGATATCCACA CAT
MS1627.Seq GAGATTTGATGAGGAGATG GTTGTGATGAGCTTCCCACTC AAAGCTTTAGGATATCCACA CAT
605 610 615 620 625 630 635 640 645 650 655 660

FIGURE 8

CGAACAGACACACACACAGAGAGGTGAATGTTAGGACCTTGCAAGTTTA
CTCAAAAAACAGCACTCTTTTATATCATGGTTTACATAAGCATTACATAAGA
CTTGGATAAGTTCCAAAAGAACATAGGAGAATAGAACACTCAGAGCTTAGAT
CAAAACATTTGATACCAAACCAAGTCAGGAAACCACTTGTCTCACATCCTTG
TTTTAAGAACAGTTTGTGACCCTGAACCTTAAACCTTGGGAACCGCAAN
GTTGGGCTCATAAAGGTTATCCATTATAGCTCATGCCAAAATTATCTGCAGA
AATGTGTTCTTAATTGTCTAGCCACTGCCCCCTCCCTTGGTATAATGAAAT
CTTTCCCCCAACGTTTCATCCCACTCCCCTAGATAAATATAATCATGTACCTGT
TGTTTTATGTCTCTTTTTCTTCTGAGTTAACACACACCAAGGAGGTCTAGC
TCTGGCGAGTCTTTCACGAAAGGGGAGGGATCTGTACAACACTTTATAGCC
GTTGACTGTGACCCACCTATCGAAATTTAAATCGTATCTTCTGTATATGGTA
GCGGGGCGTCTGTTGGTCTGTAGATGTAAGTCCCGGTTGCCAACCTGTCTC
TCCTATTTTGACAAGCGTACTCTCTTCCCCTTTTTACTTCTAGGCCCTGAGG
CCCTTAGTCTTGCACCTGTTCTTCAACTGAGGTTGAGCGTCTCTTTCTATTT
TCTATTCCCATTTCTAACCTTTGAATTTGAGTAAATATAGTGCTAAAAGACAA
AGATTCATTTCTTAACATCATGATTAATAATCGACCTATTGGATTGGTCTTATT
GGTAAAAATATAATTTTAGCAAGCATTCTTATTTCTATTTCTGAAGGACAAA
GTCCGGTGTGGCTTGTAANAGGAANTTGGCTGTGGTCTTGCCCCACGAGGA
AGGTCGAGTTCTCCGAATTGTTTAGATTGTAATCTTGACAGAGAAGAGTTATTA
AAAGAATCAAGGGTGAGAGCCCTGCGAGCACGAACCGCAACTTCCCCCAAT
AGCCCCAGGCAAAGCAGAGCTATGCCAAGTTTGCAGCAGANAATGAGTATG
TCTTTGTCTGATGGGCTCATCCGCGTGCACGCAGACGGGTCTGCTCTGGTG
GGAAACAACCCCTTGGCTGCTTCTCTCTAAGTGTAGGACACTCTCGGGAG
TTCAACCATTTCTGCTGCAGGCGCGGCATTCCCCCTTTTTCTTTTTTAAAA
GAAGCACGTTAAGATCTGACTGCACCTTGGTCAAGGCTCTTCGCAAAGCACT
GGAAAATAACGGGGGAAAATCATAAGTACTATGACCAAAGCAGGGCTCCAA
CTCCTATAAAAATGAAATATTGTGTTCTAATCCAATGGATTTAAAGCCTTTAC
TCCATTGGCNAAGGANTGANCCAACCCCTGAGGTCCCTGCGTTCAAATTTTT
TTGCTCNTATCCTAATCCAATTGGTAACCCCGTTTTNTTTTTGAAACTCATGTC
TTCAAATGCCCAATAAATGAGCCCTGGTTCTTTCCCAGCTCTCAGAAGCATT
ATACGGNANAGGTGTGACACAGCATAAAATCATAATTTGCATGACACCTAGT
GGACATTCTGGTCTTTAAGTTTGCCACATCTTGTCCTCAACTCTAAAACCTACTT
CTTCTAAAGCATTAAAGTCTAGCTTTCAATTTAAGTCTATTATTCTTTGTTTCA
ATNAGGCTAATGTAACATTTCTATGAAGATTATTAACAAACGTAGCAGTTTGC
ATCTCCTTAACCTAAGGCAGTAGTAGCTACAGCAAAGGAAGTGATAATAGCAA
TTAAAGCAGATATGCCCAGAATAATGGCAGCGACGAATCGCTTAGCTCGAAT
TAAATCTGTGGCATAACCTAAAGGTTTGAATGGCAGAATCATCAAACCATGGT
TCATCACCAATATCTACAGGTTACAACACATATGGCGGCCCTTGAATATGA
ATCGCTGCATATCCGTNNGGCAAAAAATCTAACCATTATTCCTCCTNCCNAAA
AACGGGATTTGAAANTTATNCCCCTTNCCCCNAACCCANACCGAGGTACCC
CATAATGNNGGGGGGTATCTANAANAGGGCATAGGGGTAAGAAAAACGGCA
GAGNNGGATCNTTTATGTTTCNGGAAATTCNNGGTTTGGGAGAATAAGATTCT
GGAGGCTGCAAATTAAGGGAAACATTNTGTATGGGGAATAGAGCAGTAAAA
TCTCTATCATGGGGATCTTTAGGGAGAATTTCCCAGGAACCAAGTAGGTTT
NAACCCATCNTGCTTCATACCATCGATGAACNTCTTTATTGACAGGGGGAGT
ATAATTTCCAAATAGATCCTTTTTGTTTTTAACTCTGATCTGACTGATCTACACT
AGGCGGGGGGAAGGGGAGAAATCCCAAAGTAACCCAAGGGCCCCCTTTGGAG
AAAAACTCACCCCTGGTCAGGGAAGGCGCAAGGCAACCCACCGTGGGAGG
GCAGACTCGTCTCCCTCCCAGAAGGCGTCTTCTTAAAGGCGATCTGGAGG
AGCAGACTCGTCTCCCTCCCAGAAGGCGTCTTCTTAAAGGCGATCTGG

FIGURE 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17877**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet.

US CL : 435/6, 5, 91.2, 7.1, 7.2; 536 23.1, 24.3, 24.33; 530/388.1, 300

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 5, 91.2, 7.1, 7.2; 536 23.1, 24.3, 24.33; 530/388.1, 300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	REDMOND et al. Sequence and expression of the mouse mammary tumour virus env gene. The EMBO Journal. 1983, Volume 2, Number 1, pages 125-131. See entire document.	1-20
A	FAFF et al., Retrovirus-like particles from the human T47D cell lines are related to mouse mammary tumour virus and are of human endogenous origin. Journal of General Virology. 21 May 1992, Volume 73, pages 1087-1097. See abstract.	1-20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 04 FEBRUARY 1997	Date of mailing of the international search report 18 MAR 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DIANNE REES <i>Jab for</i> Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17877

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CREPIN et al. Sequences Related to Mouse Mammary Tumor Virus Genome in Tumor Cells and Lymphocytes from Patients with Breast Cancer. Biochemical and Biophysical Research Communications. 13 January 1984, Volume 118, Number 1, pages 324-331. See entire document.	1-20
A	MESA-TEJADA et al. Detection in human breast carcinomas of an antigen immunologically related to a group-specific antigen of mouse mammary tumor virus. Proceedings of the National Academy of Sciences, USA. March 1978, Volume 75, Number 3, pages 1529-1533.	1-20

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/17877

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C12Q 1/68, 1/70; C12P 19/34; C07H 21/02, 21/04; G01N 33/53; C07K 15/28; 5/00

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, BIOTECHABS, BIOTECHDS, CABA, CAPLUS, CANCERLIT, DGENE, DRUGU, EMBASES, MEDLINE, USPATFULL, TOXLIT, TOXLINE, JAPIO, WPIDS
search terms: MMTV, mouse mammary tumor virus, PCR, hybridization, antibodies, immunoassays, Westerns, searched SEQ. ID. Nos.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: C12Q 1/68, 1/70, C12P 19/34, C07H 21/02, 21/04, G01N 33/53, C07K 15/28, 5/00	A1	(11) International Publication Number: WO 97/17470 (43) International Publication Date: 15 May 1997 (15.05.97)
(21) International Application Number: PCT/US96/17877 (22) International Filing Date: 8 November 1996 (08.11.96) (30) Priority Data: 08/555,394 9 November 1995 (09.11.95) US (71)(72) Applicant and Inventor: HOLLAND, James, F. [US/US]; 31 Mamaroneck Road, Scarsdale, NY 10583 (US). (72) Inventor: POGO, Beatriz, G., T.; 237 Nyac Avenue, Pelham, NY 10803 (US). (74) Agents: CLARK, Richard, S. et al.; Brumbaugh, Graves, Donohue & Raymond, 30 Rockefeller Plaza, New York, NY 10112 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: DETECTION OF MAMMARY TUMOR VIRUS-LIKE SEQUENCES IN HUMAN BREAST CANCER (57) Abstract The present invention relates to materials and methods for diagnosing breast cancer in humans. It is based, at least in part, on the discovery that a substantial percentage of human breast cancer tissue samples contained nucleic acid sequences corresponding to a portion of the mouse mammary tumor virus <u>env</u> gene. In contrast, such sequences were absent in almost all other human tissues tested.		

* (Referred to in PCT Gazette No. 31/1997, Section II)

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Description

Detection Of Mammary Tumor Virus-Like Sequences In Human Breast Cancer

Cross-Reference to Related Application

This application is a continuation-in-part application of U.S. Serial No. 08/555,394, filed November 9, 1995.

Statement Regarding Federally Sponsored Research

- 5 This invention was made with funds from the U.S. government, which has certain rights in the invention.

Introduction

- 10 The present invention relates to materials and methods for diagnosing breast cancer in humans. It is based, at least in part, on the discovery that a substantial percentage of human breast cancer tissue samples contained nucleic acid sequences corresponding to a portion of the mouse mammary tumor virus env gene. In contrast, such sequences were absent in almost all other human tissues tested.
- 15

Background of the Invention

- A large body of information has accumulated about the molecular biology of MMTV (reviewed in Slagle, B.L. et al., 1987, in "Cellular and Molecular Biology of Mammary Cancer", Kidwell et al., eds., Plenum Press, NY. pp 275-306). Mouse mammary tumor virus (MMTV) is associated with a high incidence of breast cancer in certain strains of mice (over 90% among females), and has been regarded as a potential model for human disease.
- 20
- 25

The MMTV virus does not carry a transforming oncogene, but rather acts as an insertional mutagen with several proviral insertion loci designated int-1

or wnt-1 (Nusse R. et al., 1982, Cell 31:99-109) int-2
(Peters, G. et al., 1983, Cell 33:369-377) int-3
(Gallahan, D. et al., 1987, J. Virol. 61:218-220) int-4
(Roelink, H. et al., 1990, Proc. Natl. acad. Sci. USA
5 87:4519-4523) and int-5 (Morris, V.L., et al. 1991,
Oncogene Research 6:53-63), which encode for growth
factors or other related proteins. These genes are not
expressed in normal mammary tissue but become activated
after integration of MMTV provirus into the adjacent
10 chromosomal DNA.

The human homolog of the int-2 locus has been
located on chromosome 11 (Casey, G. et al., 1986,
Mol. Cell Biol. 6:502-510) and has been found amplified
(in 15% of the breast cancers) and also expressed
15 (Lidereau, R. et al., 1988, Oncogene Res 2:285-291;
Zhou, D.J. et al., 1988, Oncogene 2:279-282; Liscia,
D.S. et al., 1989, Oncogene 4:1219-1224; Meyers, S.L.
et al., 1990, Cancer Res 50:5911-5918). It may be
significant that in tumors from Parsi women, who have a
20 high incidence of breast tumors, the int-2 locus is
amplified in 50% of the cases (Barnabas-Sohi, N. et
al., 1993, Breast Dis. 6:13-26). The amplification of
int-2 and other genes in 11q13 is indicative of poor
prognosis (Schuwring, E. et al., 1992, Cancer Research
25 52:5229-5234; Champeme, M-H, et al., 1995, Genes,
Chromosomes and Cancer 12:128-133). Both mouse and
human int-2 have been sequenced (Moore, R. et al.,
1986, EMBO J 5:919-924). The gene encodes a protein of
about 27 kilodaltons (KD) which shows homology to both
30 basic and acidic fibroblast growth factors (Dickson, C.
et al. 1987, Nature (London) 326:833).

However, efforts to demonstrate the presence of
viruses in human breast cancer through search for viral
particles, immunological cross-reactivity, or sequence
35 homology have yielded contradictory results. Detect-
able MMTV env gene-related antigenic reactivity
has been found in tissue sections of breast cancer

(Mesa-Tejada et al., 1978, Proc. Natl. Acad. Sci. USA 75:1529-1533; Levine, P. et al., 1980, Proc. Am. Assoc. Cancer Res. 21:170; Lloyd, R. et al., 1983, Cancer 51:654-661), breast cancer cells in culture (Litvinov, S.V. and Golovkina, T.V., 1989, Acta Virologica 33:137-142), human milk (Zotter S. et al., 1980, Eur. J. Cancer 16:455-467) in sera of patients (Day, N.K. et al., 1981, Proc. Natl. Acad. Sci. USA 78:2483-2487), in cyst fluid (Witkin, S.S. et al., 1981, J. Clin. Invest. 67:216-222) and in particles produced by a human breast carcinoma cell line (Keydar, I. et al., 1984, Proc. Natl. Acad. Sci. USA 81:4188-4192). Sequence homology to MMTV has been found in human DNA under low stringency conditions of hybridization (Callahan, R. et al., 1982, Proc. Natl. Acad. Sci. USA 79:5503-5507) and RNA related to MMTV has been detected in human breast cancer cells (Axel, R. et al., 1972, Nature 235:32-36). The presence of MMTV related sequences in lymphocytes from patients with breast cancer has been reported (Crepin, M. et al., 1984, Biochem. Biophys. Res. Comm. 118:324-331), as well as detection of reverse transcriptase (RT) activity in their monocytes (Al-Sumidaie, A.M. et al., 1988, Lancet 1:5-8). May and Westley (May and Westley, 1989, Cancer Research 49:3879-3883) have reported the presence of MMTV-like sequences arranged as tandem repeats only in DNA from breast cancer cells.

These results have been difficult to interpret, and theories linking MMTV or a related virus with human breast cancer have fallen out of favor, in view of the relatively recent discovery of human endogenous retroviral sequences ("HERs"; Westley, B. et al., 1986, J. Virol. 60:743-749; Ono, M. et al., 1986, J. Virol. 60:589-598; Faff, O. et al., 1992, J. Gen. Virology 73:1087-1097). Data which could be interpreted to demonstrate the presence of MMTV-related sequences could be more readily explained by endogenous human

retroviral sequences. Adding further confusion to the picture, env-gene related antigenicity has been detected in epitopes of human proteins (Hareuveni, M. et al., 1990, Int. J. Cancer 46:1134-1135).

5 Brief Summary of the Invention

 The present invention relates to methods for diagnosing breast cancer in humans in which the presence of mouse mammary tumor virus env gene-like sequences bears a positive correlation to the existence of malignant breast disease. It is based, at least in part, on the discovery that 38 to 40 percent of human breast cancer tissue samples tested contained gene sequences homologous to the mouse mammary tumor virus env gene that are substantially absent from other human tumors and tissues. The invention also relates to methods for diagnosing breast cancer in humans in which the presence of retrovirus proviral fragments substantially homologous to the env gene and/or 3' LTR sequence of MMTV are detected. The molecular probes used in these experiments were designed to avoid cross-hybridization with endogenous human retroviral sequences. The present invention further provides for compositions of molecular probes which may be utilized in such diagnostic methods.

25 Brief Description of the Figures

FIGURE 1: Amplification of 660 bp of MMTV-like env gene. DNA was extracted from frozen tissues. PCR was performed using primers 1 and 3. A: 2% agarose gel electrophoresis. B: Southern blot hybridization using 5',³²P-end-labeled probe 2. Lanes 1 and 3: breast cancer; lanes 2 and 4: normal breast; lane 5: control reaction (no DNA); lane E: MMTV env gene. M: molecular weight marker. Arrow indicates 510 bp band.

FIGURE 2: Nested PCR. A: 2% agarose gel electrophoresis. 1: Amplification of 686 bp of MMTV-like env

gene sequences using primers 1 and 4 and the product of reaction A 1 as template. 2: Amplification of 250 bp of MMTV-like env gene sequences using primers 2 and 3. B, 1 and 2: Southern blot hybridization of the amplified products using probe 5'-³²P end-labeled probe 2a.

FIGURE 3: Amplification of 250 bp of MMTV-like env gene. DNA was extracted from paraffin-embedded tissue sections. PCR was performed using primers 2 and 3. A: 2% agarose gel electrophoresis. B: Southern blot hybridization using 5'-³²P-labeled probe 2a. Lane 1: normal breast; lanes 2 to 5: breast cancer; lane E: MMTV env gene. M: molecular weight marker. Arrow indicates 298 bp band.

FIGURE 4: Nucleotide sequence of the cloned MMTV env gene-like sequences as compared to the env sequences of the GR and BR6 strains of MMTV using the GCG program. *:potential glycosylation site, |:mismatch to MMTV.

FIGURE 5: Southern blot hybridization of genomic DNA. DNA was extracted from frozen tissues or cell lines, digested with EcoRI and transferred to nitrocellulose paper. Hybridization with ³²P-labeled clone 166. DNA from A, B, and G: env gene positive breast cancer; C and D: env negative breast cancer; E and F: normal breast; H: MCF-7 cells. M: molecular weight marker, Arrow indicates 9kb band.

FIGURE 6: Southern blot hybridization of genomic DNA. Experimental conditions as in Fig. 5. DNA from A and B: env negative breast cancer; C and D: env positive breast cancer; E: molecular weight marker (non-labelled); F. to H: normal breast. Arrow indicates position of 9 kb marker.

FIGURE 7: Map of MMTV.

FIGURE 8: Comparison of the nucleic acid sequence of mouse mammary tumor env gene ("MMTENV"), showing residues 976-1640, with the nucleic acid sequence of a

representative 660 bp sequence obtained by PCR reaction of DNA from human breast cancer tissue ("MS1627").

FIGURE 9: Sequence of an about 2.6 kb MMTV-like fragment detected in a human breast carcinoma.

5 Detailed Description of the Invention

The present invention relates to methods and compositions for diagnosing breast cancer in humans.

The present invention provides for compositions comprising an isolated and purified nucleic acid molecule which (i) hybridizes to a gene of mouse mammary tumor virus; (ii) is present in at least 20 percent of DNA samples prepared from breast cancer tissue of different human subjects; and (iii) is present in less than 5 percent of DNA samples prepared from tissues other than breast cancer tissue from different human subjects. A "gene of mouse mammary tumor virus" includes, but is not limited to, the gag, pol, and env genes and the 5' LTR and 3' LTR sequences of MMTV. In preferred embodiments of the invention, the mouse mammary tumor virus (hereafter "MMTV") gene is the env gene and/or the 3' LTR sequence. The term "hybridize" is used to refer to routine DNA-DNA or DNA-RNA hybridization techniques under what would be regarded, by the skilled artisan, as stringent hybridization conditions. The phrase "is present" indicates that a native form of the molecule, in an unpurified state (for example, as part of chromosomal DNA), may be detected by a standard laboratory technique, such as Southern blot or polymerase chain reaction (PCR). To be "present", the molecule may be detectable by one technique but not others. To be present in "less than 5 percent of DNA samples prepared from tissues other than breast cancer tissue from different human subjects", all non-breast cancer tissue samples are considered together, but the total number of samples must be large enough to give the 5 percent

value statistical significance that would be reasonable to the skilled artisan.

In order to identify such a nucleic acid molecule, the sequence of MMTV may be compared, using a computer database, to known human DNA sequences, and portions of MMTV which are less than or equal to 25 percent homologous to a human sequence may be selected for further study. The term "homologous", as used herein, refers to the presence of identical residues; for example, a first sequence is considered 25 percent homologous to a second sequence if it shares 25 percent of the residues of the first sequence. Since there is relatively greater likelihood that MMTV may bear similarity to human retroviral-like sequences, it may be preferable to evaluate whether a particular MMTV nucleic acid sequence is homologous to such sequences, for example, as endogenous human retrovirus sequences. A prototype of such viruses is HERV-K10 (Ono, M. et al., 1986, J. Virol. 60:589-598).

Once an MMTV gene sequence which is less than or equal to 25 percent homologous to a human DNA sequence, such as a human endogenous retroviral sequence, is identified, the presence of nucleic acid molecules having the MMTV gene sequence in human breast cancer tissues and other tissues may be evaluated. Such evaluations may be performed either by Southern blot techniques, or, preferably, by polymerase chain reaction (PCR) techniques, which are more sensitive. In such a way, MMTV gene sequences which (i) hybridize to at least 20 percent of DNA samples prepared from breast cancer tissue of different human subjects and (ii) hybridize to less than 5 percent of DNA samples prepared from human tissues other than breast cancer tissues may be identified. A nucleic acid molecule having a MMTV gene sequence which satisfies these requirements may then be used in diagnostic methods which detect the presence of such sequence in human

breast tissue by standard techniques, including PCR techniques which assay for the presence of the molecule, but also, where appropriate, Southern blot, Northern blot, or Western blot techniques, to name but
5 a few.

In preferred embodiments, the present invention relates to a portion of MMTV localized between MMTV env gene sequences 976 and 1640 (Majors, I.E. and Varmus, H.E., 1983, J. Virol. 47:495-504; see Fig. 7). This
10 about 660 bp sequence (hereafter, "the 660 bp sequence") has been found to exhibit low (16 percent) homology to the prototype human endogenous retrovirus HERV-K10, using the IBI/Pustell Sequence Analysis Program, and has also been shown to be present in 121
15 (38.5%) of 314 unselected breast cancer tissue samples, in cultured breast cancer cells, in 2 of 29 breast fibroadenomas (6.9%) and in 2 of 107 breast specimens from reduction mammoplasties (1.8%). The sequence was not found in normal tissues including breast, lympho-
20 cytes from breast cancer patients nor in other human cancers or cell lines (see example section, infra). Similarly, an about 250 bp sequence (hereafter "the 250 bp sequence"), between positions 1388 and 1640 in the env gene, and therefore falling within the 660 bp
25 sequence, was detected in 60 (39.7%) of 151 breast cancer, and in one of 27 normal breast samples assayed from paraffin-embedded sections. Cloning and sequencing of the 660 bp and 250 bp sequences demonstrated that they are 95-99% homologous to MMTV env gene, but
30 not to the known human endogenous retroviruses ("HERs") nor to other viral or human genes (<18%).

In another preferred embodiment, the present invention relates to a nucleic acid molecule which corresponds to a retroviral genomic fragment which has
35 substantial homology to 3' LTR and/or env gene of the MMTV genome, and is found in a substantial percentage of breast cancer samples. By substantial percentage is

meant at least 20% of tested breast cancer samples. Such a sequence is preferably comprised of the 3' LTR region and all or part of the env gene, although it may include more sequences of a retroviral genome. Most
5 preferably, the sequence is at least comprised of an about 2.6 kb fragment which comprises the 1,228 base pair (bp) sequence of the 3' LTR sequence and 1,336 bp of the env gene sequence of MMTV (Fig. 9) (SEQ ID NO:20). When compared with the two strains of MMTV C3H
10 and BR6, the sequence homology was 90.8% and 90.7%, respectively. When compared with the endogenous retroviral sequences (HUMERKA), sequence homology was only 58% in 36 bp and 71% in 74 bp.

Retrovirus proviral sequences can be detected by
15 PCR technology using primers derived from the MMTV genome. Such primers include primer 5L, containing the nucleotides 7376-7395 of the MMTV BR6 genome (5'-3': CCAGATCGCCTTTAAGAAGG) (SEQ ID NO:11) and primer LTR3, containing nucleotides 9918-9927 of the MMTV BR6 genome
20 (5'-3': CGAACAGACACAAAGCGACG) (SEQ ID NO:19). Other primers which correspond to or are homologous to MMTV sequences can be used as primers. Nucleotide fragments which correspond to or are homologous to the retroviral sequences isolated from the breast cancer samples can
25 also be used to amplify additional retroviral fragments from the samples. Long PCR techniques can be used to amplify longer stretches of a proviral sequence.

The present invention provides for compositions comprising an isolated and purified nucleic acid
30 molecule which hybridizes to the about 2.6 kb retroviral fragment shown in Fig. 9 under stringent conditions or is at least 90 percent homologous to said fragment using the MacVector homology determining program which may be used to diagnose breast cancer in
35 a subject, using methods which include PCR and Southern blot methods.

Nucleic acids having the 660 bp sequence, the 250 bp sequence, or all or part of the about 2.6 kb sequence, may therefore be used, according to the invention, to diagnose breast cancer in a subject, using methods which include PCR and Southern blot methods. Where PCR methods are used, primers such as those listed in Table 1, below, may be utilized.

The present invention provides for compositions comprising essentially purified and isolated nucleic acid having the 660 bp sequence or the 250 bp sequence or an at least five bp, and preferably greater than or equal to ten bp, subsequence thereof. In order to maintain the desired specificity, such nucleic acid molecules may preferably contain sequence falling within the 660 bp sequence, but preferably do not contain sequences from other portions of the MMTV genome, which may, undesirably, hybridize to human sequences which are not breast cancer specific, such as HERs. Accordingly, the present invention provides for compositions wherein the isolated and purified nucleic acid molecule comprises at least a portion having a nucleic acid sequence which hybridizes to a region of the mouse mammary tumor virus env gene between residues 976 and 1640, or between residues 1388 and 1640, and wherein the isolated and purified nucleic acid molecule does not hybridize to any other region of the MMTV genome.

The 660 bp sequence, in various embodiments, may have a number of nucleotide sequences. For example, in one embodiment, the 660 bp sequence may have a sequence as set forth in Fig. 8 and designated "MMTENV-like sequence" (SEQ ID NO:17), which depicts the MMTV env sequence between residues 976 and 1640. In a second series of embodiments, the 660 bp sequence may have a sequence as set forth in Fig. 8 and designated "MS1627" (SEQ ID NO:18), which depicts a predominant sequence for the 660 bp sequence as it has been defined by

sequencing analysis of the products of PCR reactions using DNA from human breast cancer tissues. In still further embodiments, the 660 bp sequence may have various other nucleotide sequences obtained by
5 sequencing the results of PCR reactions to detect the presence of 660 bp sequence in human breast cancer tissues.

In related embodiments, the present invention provides for compositions comprising PCR primers
10 that may be used to detect the presence of the forementioned molecules or other MMTV-like sequences. For example, the compositions may comprise one or more of the following primer molecules (5' - 3'):
CCTCACTGCCAGATC (SEQ ID NO:1); GGGAATTCCTCACTGCCAGATC
15 (SEQ ID NO:2); CCTCACTGCCAGATCGCCT (SEQ ID NO:3);
TACATCTGCCTGTGTTAC (SEQ ID NO:4); CCTACATCTGCCTGTGTTAC
(SEQ ID NO:5); CCGCCATACGTGCTG (SEQ ID NO:6);
ATCTGTGGCATACT (SEQ ID NO:7); GGGAATTCATCTGTGGCATACT
(SEQ ID NO:8); ATCTGTGGCATACTAAAGG (SEQ ID NO:9);
20 GAATCGCTTGGCTCG (SEQ ID NO:10); CCAGATCGCCTTTAAGAAGG
(SEQ ID NO:11); TACAGGTAGCAGCACGTATG (SEQ ID NO:12);
CGAACAGACACAAACACACG (SEQ ID NO:19).

The use of such compositions and molecules in PCR and Southern blot techniques is illustrated in the non-
25 limiting examples set forth below. The correlation between the presence of the MMTV-related nucleic acid molecules described above and breast cancer allows such molecules and compositions to be utilized in the diagnosis of breast cancer. Accordingly, the present
30 invention provides for a method of diagnosing breast cancer, wherein the detection of such nucleic acid molecules bears a positive correlation to the existence of breast cancer in a human. The results of such
evaluation, together with additional clinical symptoms,
35 signs, and laboratory test values, may be used to formulate the complete diagnosis of the patient.

In further related embodiments, the present invention provides for an essentially purified peptide encoded by a nucleic acid molecule which (i) hybridizes to a gene of MMTV; (ii) is present in at least
5 20 percent of DNA samples prepared from breast cancer tissue of different human subjects; and (iii) is present in less than 5 percent of DNA samples prepared from tissues other than breast cancer tissue from different human subjects. In preferred embodiments, the
10 MMTV gene is the env gene.

Such peptides may be used in the diagnosis of breast cancer. Accordingly, the present invention provides for a method of diagnosing breast cancer in a human subject, comprising detecting the presence of
15 a peptide encoded by a nucleic acid molecule which (i) hybridizes to the env gene of a mouse mammary tumor virus; (ii) is present in at least 20 percent of DNA samples prepared from breast cancer tissue of different human subjects; and (iii) is present in less than
20 5 percent of DNA samples prepared from tissues other than breast cancer tissue from different human subjects.

The present invention also provides for antibodies (including monoclonal and polyclonal) antibodies which
25 specifically bind to such peptides. Such antibodies may be used in methods of diagnosing breast cancer, for example, but not by way of limitation, by Western blot, immunofluorescent techniques, and so forth.

In nonlimiting embodiments of the invention, the
30 skilled artisan may evaluate MMTV-like nucleic acid molecules for regions which would be considered likely to encode immunogenic peptides (using, for example, hydropathy plots). Such peptides may then be sequenced and used to produce antibodies that may be employed in
35 diagnostic methods as set forth above.

For example, certain peptides encoded by portions of the 660 bp sequence have been synthesized. These

peptides, which have the sequences LKRPGFQEHMI (SEQ ID NO:13) and GLPHLIDIEKRG (SEQ ID NO:14), have been used to produce antibodies in rabbits, and the resulting antisera have successfully identified breast cancer cells positive for MMTV env-like sequences by PCR assay. Other peptides encoded by 660 bp sequence which may be useful according to the invention include TNCLDSSAYDTA (SEQ ID NO:15) and DIGDEPWFD (SEQ ID NO:16).

10 6. Example: The Detection of Mouse Mammary Tumor Virus Env Gene-Like Sequences in Human Breast Cancer Cells and Tissues

 6.1. Materials and Methods

 DNA from breast cancer tissue and other human cancer tissues, human placentas, normal human tissues including breast, and from several human cell lines (including eight breast cancer cell lines), and two normal breast cell lines was extracted following the procedure of Delli Bovi et al. (1986, Cancer Res. 46:6333-6338). The DNA was resuspended in a solution containing 0.05 M Tris HCl buffer, pH 7.8, and 0.1 mM EDTA, and the amount of DNA recovered was determined by microfluorometry using Hoechst 33258 dye (Cesarone, C. et al., 1979, Anal Biochem 100:188-197). Plasmids containing the cloned genes of MMTV were obtained from the ATCC, propagated in Escherichia coli cultures and purified using anion-exchange minicolumns (Qiagen) or by precipitation with polyethylene glycol (Sambrook J., et al., 1989, in "Molecular Cloning/A Laboratory Manual", Cold Spring Harbor). Oligonucleotide primers were synthesized at the core facilities of the Brookdale Molecular Biology Center at Mount Sinai School of Medicine.

 Polymerase chain reaction (PCR) was performed using Taq polymerase following the conditions recommended by the manufacturer (Perkin Elmer Cetus)

with regard to buffer, Mg^{2+} and nucleotide concentrations. Thermocycling was performed in a DNA cycler by denaturation at 94° C for 3 min. followed by either 35 or 50 cycles of 94°C for 1.5 min., 50° C for 2 min. and 72°C for 3 min. The ability of the PCR to amplify the selected regions of the MMTV env gene was tested by using as positive templates the cloned MMTV env gene and the genomic DNA of the MCF-7 cell line, since it was shown to express gp52 immunological determinants (Yang, N.S., et al., 1975, J. Natl. Cancer Inst. 61:1205-1208). Optimal Mg^{2+} , primer concentrations and requirements for the different cycling temperatures were determined with these templates. The master mix as recommended by the manufacturer was used. To detect possible contamination of the master mix components, a reaction without template was routinely tested. γ DNA and control primers provided by the manufacturer were used as control for polymerase activity. As an internal control, amplification of a 120 bp sequence estrogen receptor gene was assayed using primers designed and generously provided by Dr. Beth Schachter, (Mount Sinai School of Medicine, N.Y.). In addition, primers for actin 5 gene amplification were also used.

The product of the PCR was analyzed by electrophoresis in a 2% agarose gel. A 1 kb DNA ladder (Gibco BRL) was used to identify the size of the PCR product. To determine if the amplified sequences of the middle region of the 660 bp faithfully reproduced the sequences of the env gene of MMTV, an 18-mer sequence within the env gene was used as a probe for the 660 bp amplified sequence. The 18-mer probe was 5' end-labeled with ^{32}P -ATP using T4 polynucleotide kinase and purified by the NENSORB nucleic acid purification cartridge (NEN). Southern blot hybridization was performed using the conditions described by (Saiki et al., 1985, Science 230:1350-1354).

The product of the PCR (660 bp or 250 bp) was cloned directly from the reaction mixture into the TA cloning vector (Invitrogen) using the TA cloning kit and following the conditions recommended by the supplier. Direct cloning of the fragment isolated from the gel, was also performed. Plasmid DNA was purified by CsCl density gradient centrifugation or by precipitation with polyethylene glycol (Sambrook et al., 1989, in "Molecular Cloning/A Laboratory Manual", Cold Spring Harbor), restricted with HindIII and EcoRI, electrophoresed in 2% agarose gels and transferred to nitrocellulose filters. Southern blot hybridization was carried out using a 5'-terminal labeled internal probe as described above. Cloning procedures were performed in laboratories totally separate from those where PCR was carried out. Automated DNA sequencing (using Applied Technology Sequencer Model 373A) was performed in the Brookdale Molecular Biology Center. Sequence homology was determined using the IBI MacVector GenBank and GCG Programs.

To prevent contamination of the samples, processing of human tissues was performed in a laminar flow hood. DNA extractions were done in a chemical hood located in a different room from that where PCR was performed. PCR assays were assembled in a biological hood provided with ultraviolet light. Aerosol resistant tips and dedicated positive-displacement pipettes were used throughout. All equipment used for PCR (microcentrifuge, electrophoresis apparatus, pipettors) was cleaned each time with 10% sodium hypochlorite to assure DNA decontamination (Prince and Andrus, 1992, Biotechniques 12:358-36). After the initial experiments were performed, the plasmid containing the MMTV env gene was frozen and never used again, to avoid contamination. However, to detect plasmid contamination from our own env gene clones,

primers were designed to amplify plasmid sequences. All the authentic MMTV env positive samples were then tested and found negative for plasmid contamination.

Southern blotting and hybridization were performed as described (Southern, E.M., 1975, J. Mol. Biol. 98:503-517), using the 660 bp cloned sequences labeled by the random primer procedure (Feinberg, A.P., et al., 1983, Anal. Biochem. 132:6-13). Prehybridization and hybridization were performed in a solution containing 6 x SSPE, 5% Denhardt's, 0.5% SDS, 50% formamide, 100 µg/ml denaturated salmon testis DNA, incubated for 18 hrs at 42°C, followed by washings with 2 x SSC and 0.5% SDS at room temperature and at 37°C and finally in 0.1 x SSC with 0.5% SDS at 68°C for 30 min (Sambrook et al., 1989, in "Molecular Cloning/A Laboratory Manual", Cold Spring Harbor). For paraffin-embedded tissue sections the conditions described by Wright and Manos (1990, in "PCR Protocols", Innis et al., eds., Academic Press, pp. 153-158) were followed using primers designed to detect a 250 bp sequence.

6.2. Results

6.2.1. Selection of Specific MMTV Env Gene Sequences

A computer search for MMTV env gene homologous sequences was first performed, since sequence homology between the human endogenous retroviral sequences and MMTV had been described. The prototype of this group of human endogenous retroviruses is HERV-K10 (Ono, M. et al., 1986, J. Virol. 60:589-598). The sequences of the env gene of MMTV (Majors, I.E. and Varmus, H.E., 1983, J Virol 47:495-504) were aligned with sequences of the env gene of the human endogenous retrovirus HERV-K10 (Ono, M. et al., 1986, J. Virol. 60:589-598), using the IBI/Pustell Sequence Analysis Program. A region of 660 bp of low homology (16%) was localized between MMTV env gene sequences 976 and 1640 (Majors, I.E. and Varmus, H.E., 1983, J Virol 47:495-504). This

internal domain of the outer membrane of the env gene has only one glycosylation site and is highly conserved between strains. Two primers comprising 15 bp sequences at positions 976-990 (primer 1) and 1626-1640 (primer 3) were first synthesized. Later longer primers were synthesized (1N and 3N). An 18-mer sequence in the middle of the 660 bp MMTV env region (1388-1405) (primer 2) was used as a probe to identify the 660 bp sequence. A second oligomer probe was synthesized comprising the sequence 1554 to 1568 (primer 2a) to be used for hybridization when a sequence of around 250 bp (between positions 1388 and 1640) was amplified. For nested PCR reactions (Mullis, K.B. and Faloona, F.A., 1987, Meth Enzymol 155:335-350), another primer comprising sequences 1647 to 1661 (primer 4) was synthesized to be used with primer 1 in the first reaction and primers 2 and 3 in the second. Modified primers with GC clamps and extra sequences were also synthesized and used in the PCR (primers 1a and 3a). Another set of primers comprising sequences 974 to 1003 (5L) and 1558 to 1577 (3L) were subsequently developed because their T_m's matched and provided better amplification than the original primers. The sequences are represented in Table 1. All of them were productive in amplification reactions.

Tabl 1. Primer and prob sequences and location in mouse mammary tumor virus env gene

Designation	Sequence (5'-3')	Location
5		
1	CCTCACTGCCAGATC	976-990
1a	GGGAATTCCTCACTGCCAGATC	976-990
1N	CCTCACTGCCAGATCGCCT	976-993
2	TACATCTGCCTGTGTTAC	1388-1405
10	2N	CCTACATCTGCCTGTGTTAC
2a	CCGCCATACGTGCTG	1554-1568
3	ATCTGTGGCATAACCT	1640-1626
3a	GGGAATTCATCTGTGGCATAACCT	1640-1626
3N	ATCTGTGGCATAACCTAAAGG	1640-1621
15	4	GAATCGCTTGGCTCG
5L	CCAGATCGCCTTTAAGAAGG	984-1003
3L	TACAGGTAGCAGCACGTATG	1558-1577

6.2.2. Detection of MMTV-Like Env Gene Sequences in Human Breast Tumor DNA

20 PCR was performed on DNA extracted from breast cancer tissues, normal breast tissues and from the plasmid containing the env gene of MMTV, using primers 1 and 3. Photographs of the ethidium bromide stained gels of the PCR product reveal the presence of an

25 approximately 660 bp sequence in some of the tumors, (Fig. 1A, lanes 1 and 3) but not in the normal tissue samples (Fig. 1A, lanes 2 and 4). As a positive control the MMTV env gene was also amplified (Fig. 1A, lane E). Similar results were obtained with modified

30 primers 1a, 3a, 3L and 5L. Southern blot hybridization of the gel with ³²P-labeled 18-mer oligonucleotide (primer 2) indicated that this internal sequence was present in the amplified material (Fig. 1B) and that the bands in the gel were not artifactual.

35 Our initial effort was to analyze a representative sample of breast cancer specimens as well as normal

tissues and other tumors. To date 343 breast tumors have been processed, DNA extracted and PCR performed. Of these 343 tumors, 314 were carcinomas and 29 were fibroadenomas. Amplification of sequences of 660 bp was observed in 121 of the carcinomas (38.5%) and in 2 of the 29 fibroadenomas (6.9%). These sequences were confirmed to be MMTV env gene-like sequences by hybridization with the labeled specific probe containing the internal sequences. These sequences were not detected in the DNAs extracted from 20 normal organs, 23 cancers from other organs and 26 samples of blood lymphocytes including 7 from breast cancer patients whose breast specimens were positive. From 107 samples of normal breast obtained from reduction mammoplasties, 2 were positive (1.8%). In addition to DNA from lymphocytes from seven positive patients, DNA from their normal breast tissue of the operated breast was tested in 4 cases. All were negative (Table 2). Finally, DNA of the MCF-7, and ED (a cell line developed in our laboratory from the pleural effusion of a patient with an env -positive breast tumor) breast cancer cell lines were shown to contain the 660 bp MMTV env gene-like sequences (Table 3), while four other breast cancer cell lines were positive only for the 250 bp sequence (T47-D, BT-474, BT-20 and MDA-MB-231).

Table 2. Detection of MMTV env g n -like sequences in human DNA xtract d from fresh or frozen tissues

5	Sample	Number	MMTV <u>env</u> gene sequences	% Positive
	Breast Carcinomas	314	121	38.5%
	Breast Fibroadenomas	29	2	6.9%
10	Normal Breasts	107	2	1.8%
	*Normal Breasts	4	negative	
	Tumors other than breast	23	negative	
	Normal tissues	20	negative	
15	Lymphocytes	26	negative	
	**Lymphocytes	7	negative	
	* Histologically normal tissue from same breast as positive cancer.			
20	** Lymphocytes from breast cancer patients who were positive for MMTV <u>env</u> gene sequences in the tumor.			

Tabl 3. Detection of MMTV env gene-like sequences in DNA from human cell lines in cultur

	Human Cell Lines		MMTV <u>env</u> gene sequence
5	MC-7	(breast carcinoma)	positive
	T47-D	" "	negative
	BT-20	" "	negative
	MDA-MB-231	" "	negative
	ZR-75-1	" "	negative
10	SK-BR 3	" "	negative
	BT474	" "	negative
	ED	" "	positive
	MCF-10	(normal breast)	negative
	HB-447	" "	negative
15	HL-60	(promyelocytic leukemia)	negative
	K562	(erythroleukemia)	negative
	Jurkat	(T cell leukemia)	negative
	Hep 6-2	(hepatoma)	negative

The nested polymerase reaction was used in several instances to increase sensitivity and specificity, thus reducing the probability of false positives. In Fig. 2, results of a representative nested reaction are shown using primers 1 and 4 in the first reaction (Fig. 2A) and 2 and 3 for the 2nd reaction. The specificity of the reaction can be seen in the 2nd amplification (Fig. 2B).

To study a large number of samples and to be able to perform archival studies, PCR of paraffin-embedded tissue sections was also carried out. Primers 2 and 3 were used to amplify a 250 bp sequence within the 660 bp stretch when DNA was extracted from paraffin-embedded tissue sections since larger size sequences are difficult to amplify after fixation. Tumor DNA was amplified (Fig. 3A, lanes 2-5) whereas normal breast DNA was not (Fig. 3A, lane 1). The identification of

this 250 bp sequence with the MMTV-like env gene was confirmed by hybridization with an internal probe (primer 2a) as shown in Fig. 3B. Using this procedure we have analyzed 151 breast cancer samples and found that 60 (39.7%) possess the 250 bp sequence. Of the 27 normal breast samples obtained from reduction mammoplasties assayed by this procedure, one was positive (3.7%). These results, in conjunction with those obtained from lymphocytes and from normal breast tissue of patients whose breast cancer was PCR positive, indicate that MMTV-like sequences are present in a significant number of human breast cancer DNA which cannot be explained by DNA polymorphism.

6.2.3. Cloning and Sequencing of the MMTV-Like Env Gene Sequences

To find out whether there was homology to MMTV env gene throughout the whole 660 bp stretch, the product of the PCR from 8 different tumors was cloned and sequenced. In Fig. 4 the sequence of different clones comprising around 600 bp are represented, as aligned to the MMTV env gene sequence of the GR and BR6 strains (Redmon, S. and Dickson, C., 1983, EMBO J. 2:125-131). This domain of the env gene in the GR strain is 100% homologous to the C₃H strain and 98% to the BR6 strain (Majors, I.E. and Varmus, H.E., 1983, J. Virol. 47:495-504; Moore, R. et al., 1987, J. Virol. 61:480-490). Evaluation of the clones indicated that homology to MMTV env gene varied from 95% to 99%. Another seven clones comprising only 250 bp were also sequenced. Homology to MMTV env gene varied from 95% to 99% (data not shown). When compared to the human endogenous provirus HERV-K10, the homology of all the clones was less than 15%. When compared against all known viral and human genes (more than 130,000 entries) using the 1B1 MacVector GenBank and GCG programs, the highest homology recorded was 18%.

6.2.4. Southern Blot Analysis Using Cloned Sequences

To investigate whether the env gene-like sequences were present in human DNA, Southern blot hybridization was performed using the cloned sequence as probe. DNAs from normal breast tissues, env positive or negative breast tumors, tumors other than breast and breast cancer cell lines were restricted with EcoRI and in some instances with PstI, BglII or KpnI. EcoRI is a frequent cutter restriction enzyme that digests MMTV proviral DNA between env and pol genes. Four different cloned 660 bp sequences were used as probes after labeling with ³²P by random prime-labeling. Results of some of the Southern blot hybridization experiments are shown in Fig. 5. They reveal the presence of a labeled restriction fragment migrating at approximately 7-8 kb in breast cancer DNA, in ED and two fragments in MCF-7 cells. Different restriction patterns were observed with the other three enzymes. The 660 bp sequence was absent in 10 normal tissues, 10 fibroadenomas and 10 tumors from other tissues. It is important to emphasize that hybridization conditions for these experiments were stringent (as described in Section 6.1) to avoid interference with endogenous sequences that might interact with the probes.

7. Example: Detection of a Retrovirus Proviral Fragment in Human Breast Cancer Cells and Tissues

7.1. Materials and Methods

To detect longer retrovirus proviral fragments in breast cancer samples, DNA was extracted from breast cancer carcinoma tissue samples as described above in Section 6.1. Two rounds of long PCR was performed on the DNA primers 5L (SEQ ID NO:11) and LTR3 (SEQ ID NO:19). The primer 5L contains nucleotides 7370-7395 of the MMTV BR6 genome (5'-3': CCAGATCGCCTTTAAGAAGG) (SEQ ID NO:11) and primer LTR3 contains nucleotides

9918-9927 of the MMTV BR6 genome (5'-3':
CGAACAGACACAAAGCGACG) (SEQ ID NO:19). Long PCR was
performed using protocols described by the manufacturer
(Perkin Elmer, Foster City, CA). The amplified
5 retroviral fragment isolated from the breast cancer
sample was cloned into the TA cloning vector
(Invitrogen) and automated sequencing was performed
as described in Section 6.1.

7.2 Results

10 An approximately 2.6 kb retroviral fragment
containing 1,228 bp of the 3' LTR sequence and 1,336 bp
of the env gene sequence of a potential provirus was
detected in a human breast carcinoma tissue sample by
the long PCR technique using the 5L and LTR3 primers.
15 The sequence of this retroviral fragment is shown in
Fig. 9. (SEQ ID NO:20).

When compared with the two strains of MMTV C3H
and BR6, the sequence homology was 90.8% and 90.7%,
respectively, over the MMTV genomic fragment from
20 nucleotides 7370-9937. When compared with the
endogenous retroviral sequences (HUMERKA), sequence
homology was only 58% in 36 bp and 71% in 74 bp.

8. Discussion

Search for virus-related sequences in human breast
25 cancer has been hampered by great variation reported
in previous studies, by the presence of endogenous
retroviral sequences in human DNA and by the lack of
sensitivity of the methods employed. The studies
reported herein circumvent these deficiencies by
30 focusing on sequences with low homology to human
endogenous retroviruses, by investigating a large
number of tumors and several types of controls and
by using the most sensitive technology presently
available.

35 The results indicate that unique MMTV env gene
sequences were present in 38.5% of the breast cancer

samples analyzed and 39.7% of archival samples of breast cancer and that these sequences were absent in normal tissues including lymphocytes from patients with positive breast cancer and in cancers other than breast. Normal breast tissue and fibroadenomas had a low frequency (1.8 to 6.9%) of positive results. When cloned and sequenced, the sequences were found to be highly homologous to MMTV env gene, but not to the endogenous retroviral sequences. Furthermore, experiments in which the cloned amplified sequences were used for hybridization with DNA from breast cancer or normal tissues revealed that homologous DNA was only present in breast cancer DNA. The results also indicate that a human breast carcinoma sample contained an about 2.6 kb MMTV-like fragment comprised of 1,336 bp of the env gene and 1,228 bp of the 3' LTR.

The detection of MMTV env gene sequences in two fibroadenomas out of 29 and in two normal breast tissue samples out of 107 samples is of uncertain significance. Although such results could potentially be artifactual, and thus may represent false positives, they may alternatively indicate the presence of histologically unrecognized cells that were or will be neoplastic.

Ninety percent (90%) of the breast cancers tested were invasive ductal carcinomas, which reflects the prevalence of this type of neoplasm. Most patients were node-positive which is probably artifactual since it was necessary that tumor size be sufficiently large to provide an aliquot for research and tumor size correlates with node positivity.

It is unlikely that differences in homology between MMTV env gene and the cloned human sequences are generated by errors committed by the Taq polymerase. It has been estimated that the rate of nucleotide misincorporation is 1×10^{-5} per cycle (Ehrlich et al, 1991, Science 252:1643-1651) and

therefore, only a total of 0.32 nucleotides misincorporated should be expected in 660 bp after 50 cycles. The differences in homology between clones from different patients is likely to represent
5 heterogeneity of the env gene.

In contrast to earlier, ambiguous data associating MMTV-like sequences with human breast cancer, we have clearly demonstrated the existence of such sequences in breast cancer cells which cannot be explained by any
10 known human endogenous retroviral sequence. Our data do not support the results of earlier studies which indicated that, as in the mouse, MMTV-like sequences were found in lymphocytes from two patients with breast cancer (Crepin, M. et al., 1984, Biochem. Biophys. Res.
15 Comm. 118:324-331). The absence of MMTV env-like sequences in lymphocytes could reflect the fate of a unique lymphocyte subset over decades between initial encounter and the appearance of clinical breast cancer; alternatively, the human disease may differ from the
20 mouse model. Results from attempts to identify unique MMTV-like pol gene sequences have shown that they cannot be distinguished from the reverse transcriptase sequences of endogenous retroviruses (Deen, K.C. and Sweet, R.W., 1986, J. Virol. 57:422-432).

25 The origin of the MMTV env gene-like and 3' LTR-like sequences found in tumor DNA could be the result of integrated MMTV-like sequences from a human mammary tumor virus. Polymorphism of endogenous retroviral sequences is conceivable but can be ruled out because
30 these sequences were not detected in lymphocytes from the positive patients, in sections of the cancerous breast from which abnormal cells were absent, or in normal breast tissue from patients with MMTV env-like positive tumors. Recombination during tumorigenesis
35 between endogenous sequences to resemble the MMTV env genes seems highly unlikely since no known gene or viral sequence is more than 18% homologous to the

660 bp sequence. The longer about 2.6 kb MMTV-like fragment detected in a human breast carcinoma had minimal homology (58% in 36 bp and 71% in 74 bp) to endogenous human retroviral sequences. Thus, the most conservative interpretation is that our findings represent exogenous sequences from an agent similar to MMTV. Recombination between endogenous and exogenous env gene sequences are known to accelerate the development of malignancies in mice (DiFronzo, N.L. and Holland, C.A., 1993, J. Virol. 67:3763-3770). Whether the MMTV-like sequences belong to an entire acquired provirus or to an exogenous fragment integrated into endogenous sequences, is presently not known. Experiments are in progress to distinguish between these possibilities.

Several genetic alterations have been identified in human breast cancer that can be useful as markers for prevention, detection or prognosis (reviewed in Runnenbaum, I. et al., 1991, Proc. Natl. Acad. Sci. USA 88:10657-10661). The BRCA1 and BRCA2 genes have recently been described. They account for at least 5% of breast cancer and are related to familial breast cancer (Miki, Y. et al., 1994, Science 266:66-71; Wooster, R. et al., 1994, Science 265:2088-2090). We have primary evidence that familial clustering of the MMTV env gene-like sequences occurs, accounting for an even higher percentage of cancers in affected families (Holland et al. 1994, Proc. Am. Assoc. Cancer Res 35:218). The presence of MMTV-like sequences may be correlated with special clinical disease status, may provide another potential molecular marker, and may distinguish a subset of human breast cancer for which viral etiology is tenable. This has implications for epidemiology, therapy and prevention.

Various publications are cited herein, the contents of which are hereby incorporated by reference in their entireties.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: HOLLAND, JAMES

5 (ii) TITLE OF THE INVENTION: DETECTION OF MAMMARY TUMOR VIRUS-LIKE
SEQUENCES IN HUMAN BREAST CANCER

(iii) NUMBER OF SEQUENCES: 20

(iv) CORRESPONDENCE ADDRESS:

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(C) CITY: New York
(D) STATE: NY
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(F) ZIP: 10112-0228

15 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 1.5

20 (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: NOT YET ASSIGNED
(B) FILING DATE: 08-NOV-1996
(C) CLASSIFICATION:

25 (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER 08/555,394
(B) FILING DATE: 09-NOV-1995

(viii) ATTORNEY/AGENT INFORMATION:

30 (A) NAME: Kole, Lisa B
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(A) TELEPHONE: 212-408-2628
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35 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

45 (v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCTCACTGCC AGATC

15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGGAATTCCT CACTGCCAGA TC

22

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCTCACTGCC AGATCGCCT

19

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TACATCTGCC TGTGTTAC

18

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30

5 (ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCTACATCTG CCTGTGTTAC

20

(2) INFORMATION FOR SEQ ID NO:6:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
20 (vi) ORIGINAL SOURCE:
(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCGCCATACG TGCTG

15

(2) INFORMATION FOR SEQ ID NO:7:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATCTGTGGCA TACCT

15

(2) INFORMATION FOR SEQ ID NO:8:

40 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGGAATTCAT CTGTGGCATA CCT

23

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATCTGTGGCA TACCTAAAGG

20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GAATCGCTTG GCTCG

15

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCAGATCGCC TTTAAGAAGG

20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TACAGGTAGC AGCACGTATG

20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Leu Lys Arg Pro Gly Phe Gln Glu His Glu Met Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Gly Leu Pro His Leu Ile Asp Ile Glu Lys Arg Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide
 (iii) HYPOTHETICAL: NO
 (iv) ANTISENSE: NO
 (v) FRAGMENT TYPE: N-terminal
 (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Thr Asn Cys Leu Asp Ser Ser Ala Tyr Asp Thr Ala
 1 5 10

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide
 (iii) HYPOTHETICAL: NO
 (iv) ANTISENSE: NO
 (v) FRAGMENT TYPE: N-terminal
 (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Asp Ile Gly Asp Glu Pro Trp Phe Asp Asp
 1 5 10

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 662 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
 (iii) HYPOTHETICAL: NO
 (iv) ANTISENSE: NO
 (v) FRAGMENT TYPE:
 (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

35	TCCTCACTGC	CAGATCGCCT	TTAAGAAGGA	CGCCTTCTGG	GAGGGAGACG	AGTCTGCTCC	60
	TCCACGGTGG	TTGCCCTTGC	CCTTCCCTGA	CCAAGGGGTG	AGTTTTTCTC	CAAAAGGGGC	120
	CCTTGGGTTA	CTTTGGGATT	TCTCCCTTCC	CTCGCCTAGT	GTAGATCAGT	CAGATCAGAT	180
	TAAAAGCAAA	AAGGATCTAT	TTGGAAATTA	TACTCCCCCA	GTCAATAAAG	AGGTTTCATCG	240
	ATGGTATGAA	GCAGGATGGG	TAGAACCTAC	ATGGTTCTGG	GAAAATTCTC	CTAAGGATCC	300
40	CAATGATAGA	GATTTTACTG	CTCTAGTTCC	CATACAGAAT	TGTTTCGCTT	AGTTGCAGCC	360
	TCAAGATATC	TTATTCTCAA	AAGGCAGGAT	TTCAGGAACA	TGAGATGATT	CCTACATCTC	420
	TGTGTTACTT	ACCCCTATGT	CATATTATTA	GGATTACCTC	AGCTAATAGA	TATAGAGAAA	480
	GAGGATCTAC	TTTTCATATT	TCCTGTTCTT	CTTGTAAGATT	GACTAATTGT	TTAGATTCTT	540
	CTGCCTACGA	CTATGCAGCG	ATCATAGTCA	AGAGGCCGCC	ATACGTGCTG	CTACCTGTAG	600
45	ATATTGGTGA	TGAACCATGG	TTTGATGATT	CTGCCATTCA	AACCTTTAGG	TATGCCACAG	660
	AT						662

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 663 base pairs
 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

10	TCCTCACTGN	CAGATCGCCT	TTAAGAAGGA	CGCCTTCTGG	GAGGGAGACG	AGTCTGCTCC	60
	TCCACGGTGG	TTGACTTGCG	CCTTCCCTGA	CCAGGGGGTG	AGTTTTTCTC	CAAAAGGGGC	120
	CCTTGGGTTA	CTTTGGGATT	TCTCCCTTCC	CTCGCCTAGT	GTAGATCAGT	CAGATCAGAT	180
	TAAAAGCAAA	AAGGATCTAT	TTGGAAATTA	TACTCCCCCT	GTCAATAAAG	AGGTTTCATCG	240
	ATGGTATGAA	GCAGGATGGG	TAGAACCCTAC	ATGGTTCTGG	GAAAATTCTC	CTAAGGATCC	300
	CAATGATAGA	GATTTTACTG	CTCTAGTTCC	CATACAGAAT	TGTTTCGCTT	AGTTGCAGCC	360
15	TCAAGATATC	TTATTACAA	AAGGCAGGAT	TTCAAGAACA	TGACATGAAT	CCCTACATCT	420
	CTGTGTTACT	TACCCTTATG	CCANANTATT	AGGATTACCT	CAGCTAATAG	ATATAGAGGA	480
	AGAGGATCTA	CTTTTCATAT	TTCCTGTTCT	TCTTGTAGAT	TGACTAATTG	TTTAGATTCT	540
	TCTGCCTACG	ACTATGCAGC	GATCATAGTC	AAGAGGCCGC	CATACGTGCT	GCTACCTGTA	600
20	GATATTGGTG	ATGAACCATG	GTTTGATGAN	NCTGCCANTC	AAACCTTTAG	GTATNCCACA	660
	GAT						663

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGAACAGACA CAAACACACG

20

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2598 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

	CGAACAGACA	CAAACACACG	AGAGGTGAAT	GTTAGGACTG	TTGCAAGTTT	ACTCAAAAAA	60
	CAGCACTCTT	TTATATCATG	GTTTACATAA	GCATTACAT	AAGACTTGGA	TAAGTTCCAA	120
	AAGAACATAG	GAGAATAGAA	CACTCAGAGC	TTAGATCAAA	ACATTTGATA	CCAAACCAAG	180
5	TCAGGAAACC	ACTTGTCTCA	CATCCTTGTT	TTAAGAACAG	TTTGTGACCC	TGAACCTACT	240
	TAAACCTTGG	GAACCGCAAN	GTTGGGCTCA	TAAAGGTTAT	CCATTATAGC	TCATGCCAAA	300
	ATTATCTGCA	GAAATGTGTT	CCTAATTGTC	TAGCCACTGC	CCCCCTCCCT	GGTATAATGA	360
	AAATCTTTCC	CCCAACGTTT	ATCCCCTCTC	CCTAGATAAA	TATAATCATG	TACCTGTTGT	420
	TTTATGTCGT	CTTTTTCTTC	CTGAGTTAAC	ACACACCAAG	GAGGTCTAGC	TCTGGCGAGT	480
10	CTTTCACGAA	AGGGGAGGGA	TCTGTACAAC	ACTTTATAGC	CGTTGACTGT	GACCCACCTA	540
	TCGAAATTTA	AATCGTATCT	TCCTGTATAT	GGTAGCGGGG	CGTCTGTTGG	TCTGTAGATG	600
	TAAGTCCCGG	TTGCCACCAC	CTGTCTCCTA	TTTTGACAAG	CGTACTCCTC	TTTCCCCTTT	660
	TTACTTCTAG	GCCTGAGGCC	CTTAGTCCTT	GCACCTGTTT	TTCAACTGAG	GTTGAGCGTC	720
	TCTTTCTATT	TTCTATTCCC	ATTTCTAACC	TTTGAATTTG	AGTAAATATA	GTGCTAAAAG	780
15	ACAAAGATTC	ATTTCTTAAC	ATCATGATTA	ATAATCGACC	TATTGGATTG	GTCTTATTGG	840
	TAAAAATATA	ATTTTCTAGC	AGCATTCTTA	TTTCTATTTT	TGAAGGACAA	AGTCGGTGTG	900
	GCTTGTAANA	GGAANTTGGC	TGTGGTCCTT	GCCCCACGAG	GAAGGTCGAG	TTCTCCGAAT	960
	TGTTTAGATT	GTAATCTTGC	ACAGAAGAGT	TATTAAGAAG	ATCAAGGGTG	AGAGCCCTGC	1020
	GAGCACGAAC	CGCAACTTCC	CCCAATAGCC	CCAGGCAAAG	CAGAGCTATG	CCAAGTTTGC	1080
20	AGCAGANAAT	GAGTATGTCT	TTGTCTGATG	GGCTCATCCG	CGTGACGCA	GACGGGTCGT	1140
	CCTTGGTGGG	AAACAACCCC	TTGGCTGCTT	CTCTCCTAAG	TGTAGGACAC	TCTCGGGAGT	1200
	TCAACCATTT	CTGCTGCAGG	CGCGGCATTT	CCCCCTTTT	TCTTTTAA	AAGAAGCACG	1260
	TTAAGATCTG	ACTGCACTTG	GTCAAGGCTC	TTCGCAAAGC	ACTGGAAAAT	AACGGGGAAA	1320
	ATCATAAGTA	CTATGACCAA	AAGCAGGGCT	CCAACCTCCTA	TAAAAATGAA	ATATTGTGTT	1380
25	CTAATCCAAT	GGATTTAAAG	CCTTTACTCC	ATTGGCNAAG	GANTGANCCA	ACCCCTGAGG	1440
	TCCCTGCGTT	CAAATTTTTT	TGCTCNTATC	CTAATCCAAT	TGGTAACCCC	GTTTNTTTTT	1500
	GAAACTCATG	TCTTCAAATG	CCCAATAAAT	GAGCCCTGGT	TCTTTCCCAG	CTCTCAGAAG	1560
	CATTATACGG	NANAGGTGTG	ACACAGCATA	AAATCATAAT	TTGCATGACA	CCTAGTGGAC	1620
	ATTCTGGTCT	TTAAGTTTGC	CACATCTTGT	CCCAACTCTA	AAACTACTTC	TTCTAAAGCA	1680
30	TTAAGTCTAG	CTTTCAATTT	TAAGTCTATT	ATTCTTTGTT	CAGATNAGGC	TAATGTAACA	1740
	TTTCTATGAA	GATTATTAAC	AAACGTAGCA	GTTTGCATCT	CCTTAACTAA	GGCAGTAGTA	1800
	GCTACAGCAA	AGGAAGTGAT	AATAGCAATT	AAAGCAGATA	TGCCCAGAAT	AATGGCAGCG	1860
	ACGAATCGCT	TAGCTCGAAT	TAAATCTGTG	GCATACCTAA	AGGTTTGAAT	GGCAGAATCA	1920
	TCAAACCATG	GTTTCATCACC	AATATCTACA	GGTTACAACA	CATATGGCGG	CCCCTTGAAT	1980
35	ATGAATCGCT	GCATATCCGT	NGGCAAAAAA	TCTAACCATT	ATTCCCTCCTN	CCNAAAAACG	2040
	GGATTTGAAA	NTTATNCCCC	TTNCCCCNAA	CCCANACCGA	GGTACCCCAT	AATGNNGGGG	2100
	GTATCTANAA	NAGGGCATAG	GGGTAAGAAA	AACGGCAGAG	NGGGATCNTT	TATGTTCTNGG	2160
	AAATTCNNGG	TTTGGGAGAA	TAAGATTCTG	GAGGCTGCAA	ATTAAGGGAA	ACATTNTGTA	2220
	TGGGGAATAG	AGCAGTAAAA	TCTCTATCAT	GGGGATCTTT	AGGGAGAATT	TTCCCAGGAA	2280
40	CCAAGTAGGT	TCNAACCCAT	CNTGCTTCAT	ACCATCGATG	AACNTCTTTA	TTGACAGGGG	2340
	GAGTATAATT	TCCAAATAGA	TCCTTTTGT	TTTTAATCTG	ATCTGACTGA	TCTACACTAG	2400
	GCGGGGGAAG	GGAGAAATCC	CAAAGTAACC	CAAGGGCCCC	TTTTGGAGAA	AAACTCACCC	2460
	CCTGGTCAGG	GAAGGCGCAA	GGCAACCACC	GTGGAGGAGC	AGACTCGTCT	CCCTCCCAGA	2520
45	AGGCGTCCTT	CTTAAAGGCG	ATCTGGAGGA	GCAGACTCGT	CTCCCTCCCA	GAAGGCGTCC	2580
	TTCTTAAAGG	CGATCTGG					2598

Claims

- 1 1. A composition comprising an oligonucleotide primer
2 which may be used to detect the presence of a
3 nucleic acid molecule which (i) hybridizes to the
4 env gene of a mouse mammary tumor virus; (ii) is
5 present in at least 38 percent of DNA samples
6 prepared from breast cancer tissue of different
7 human subjects; and (iii) hybridizes to less than
8 7 percent of DNA samples prepared from tissues
9 other than breast cancer tissue from different
10 human subjects.
- 1 2. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 CCTCACTGCCAGATC (SEQ ID NO:1).
- 1 3. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 GGGAATTCCTCACTGCCAGATC (SEQ ID NO:2).
- 1 4. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 CCTCACTGCCAGATCGCCT (SEQ ID NO:3).
- 1 5. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 TACATCTGCCTGTGTTAC (SEQ ID NO:4).
- 1 6. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 CCTACATCTGCCTGTGTTAC (SEQ ID NO:5).
- 1 7. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 CCGCCATACGTGCTG (SEQ ID NO:6).

- 1 8. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 ATCTGTGGCATACT (SEQ ID NO:7).
- 1 9. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 GGAATTCATCTGTGGCATACT (SEQ ID NO:8).
- 1 10. The composition of claim 1, wherein the
2 oligonucleotide primer comprises a sequence
3 selected from the group consisting of
4 ATCTGTGGCATACTAAAGG (SEQ ID NO:9);
5 GAATCGCTTGGCTCG (SEQ ID NO:10);
6 CCAGATCGCCTTTAAGAAGG (SEQ ID NO:11); and
7 TACAGGTAGCAGCACGTATG (SEQ ID NO:12).
- 1 11. An essentially purified peptide encoded by a
2 nucleic acid molecule which (i) hybridizes to
3 a gene of MMTV; (ii) is present in at least
4 20 percent of DNA samples prepared from breast
5 cancer tissue of different human subjects; and
6 (iii) is present in less than 5 percent of DNA
7 samples prepared from tissues other than breast
8 cancer tissue from different human subjects.
- 1 12. An antibody which specifically binds to the
2 peptide of claim 11.
- 1 13. The peptide according to claim 11 which comprises
2 the amino acid sequence LKRPGFQHEMI (SEQ ID
3 NO:13).
- 1 14. An antibody which specifically binds to the
2 peptide of claim 13.
- 1 15. The peptide according to claim 11 which comprises
2 the amino acid sequence GLPHLIDIEKRG (SEQ ID NO:14).

- 1 16. A method of diagnosing breast cancer in a human
2 subject, comprising detecting the presence of a
3 peptide encoded by a nucleic acid molecule which
4 (i) hybridizes to the env gene of 3' LTR of a
5 mouse mammary tumor virus; (ii) is present in at
6 least 20 percent of DNA samples prepared from
7 breast cancer tissue of different human subjects;
8 and (iii) is present in less than 5 percent of DNA
9 samples prepared from tissues other than breast
10 cancer tissue from different human subjects.
- 1 17. The method according to claim 16, wherein the
2 presence of a peptide comprising the amino acid
3 sequence LKRPGFQEHEMI (SEQ ID NO:13) is detected
4 by the binding of an antibody specific to the
5 peptide.
- 1 18. The method according to claim 16, wherein the
2 presence of a peptide comprising the amino acid
3 sequence GLPHLIDIEKRG (SEQ ID NO:14) is detected
4 by the binding of an antibody specific to the
5 peptide.
- 1 19. The method according to claim 16, wherein the
2 presence of a peptide comprising the amino acid
3 sequence TNCLDSSAYDTA (SEQ ID NO:15) is detected
4 by the binding of an antibody specific to the
5 peptide.
- 1 20. The method according to claim 16, wherein the
2 presence of a peptide comprising the amino acid
3 sequence DIGDEPWFD (SEQ ID NO:16) is detected by
4 the binding of an antibody specific to the
5 peptide.
- 1 21. A composition comprising an oligonucleotide primer
2 which may be used to detect the presence of a

3 nucleic acid molecule which (i) hybridizes to a
4 nucleic acid comprised of a sequence selected from
5 the group consisting of the env gene and the 3'
6 LTR of a mouse mammary tumor virus; (ii) is
7 present in a substantial percentage of DNA samples
8 prepared from breast cancer tissue of different
9 human subjects; and (iii) hybridizes to less than
10 5 percent of DNA samples prepared from tissues
11 other than breast cancer tissue from different
12 human subjects.

1 22. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 CCAGATCGCCTTTAAGAAGG (SEQ ID NO:11).

1 23. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 CGAACAGACACAAACACACG (SEQ ID NO:19).

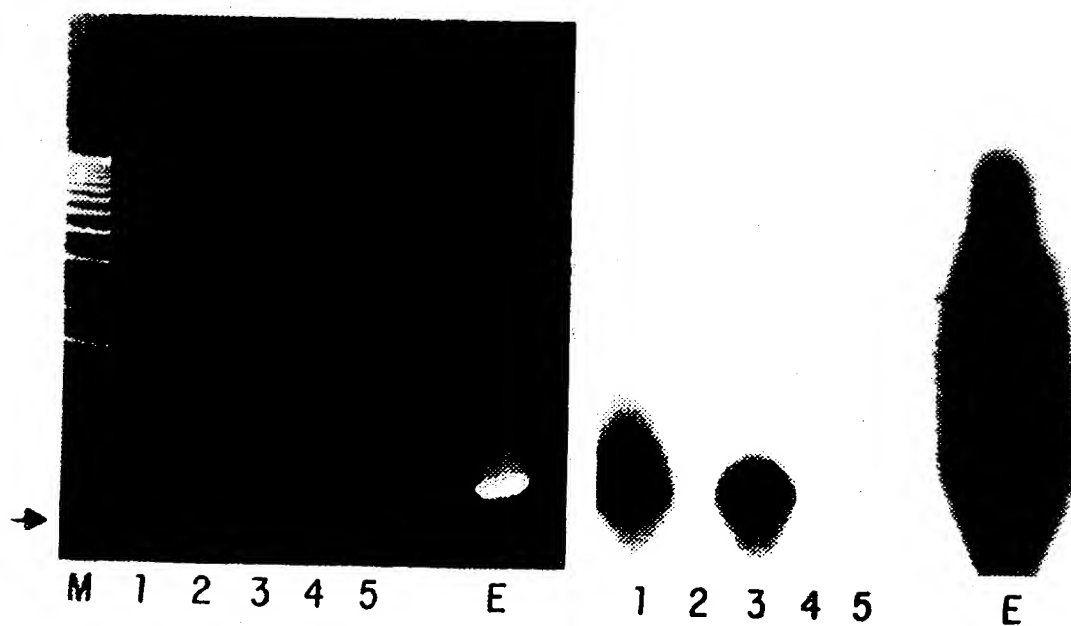


FIG. 1A

FIG. 1B

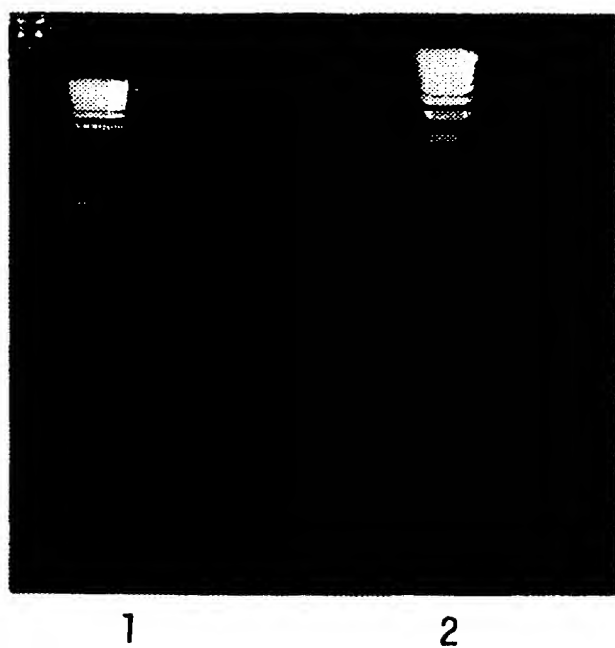


FIG. 2A

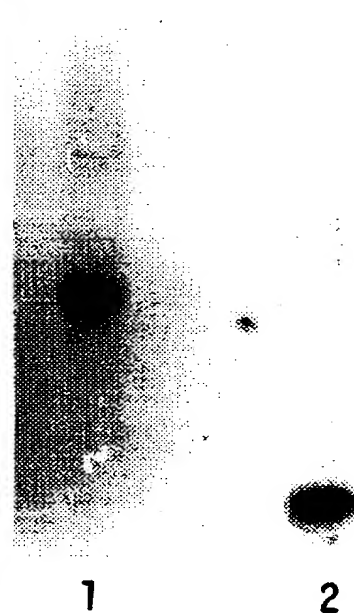
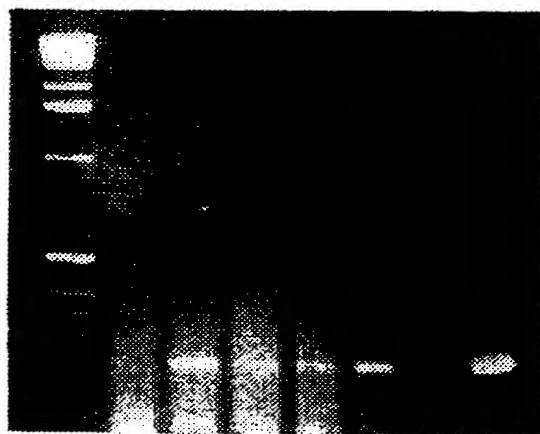
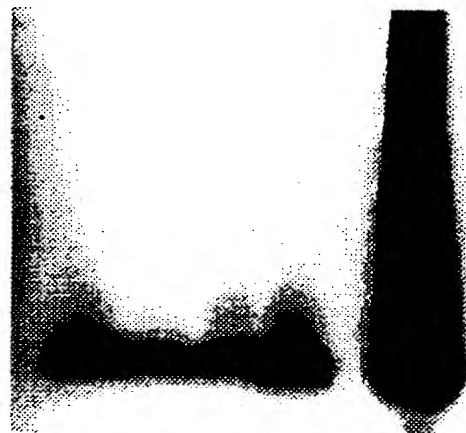


FIG. 2B



M 1 2 3 4 5 E

FIG.3A



1 2 3 4 5 E

FIG.3B

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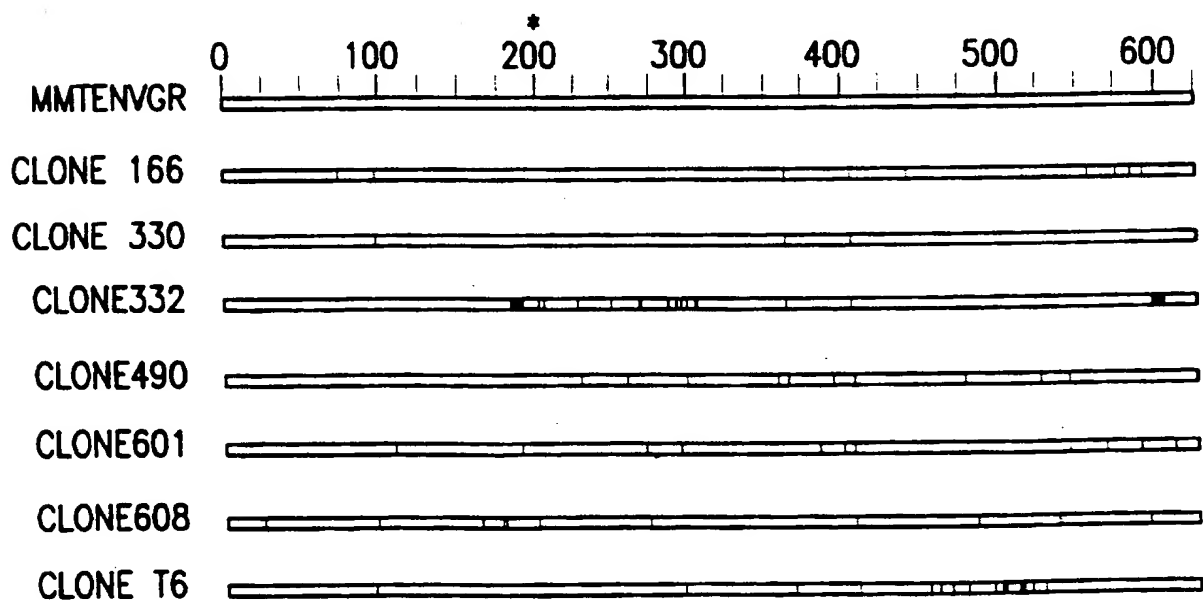
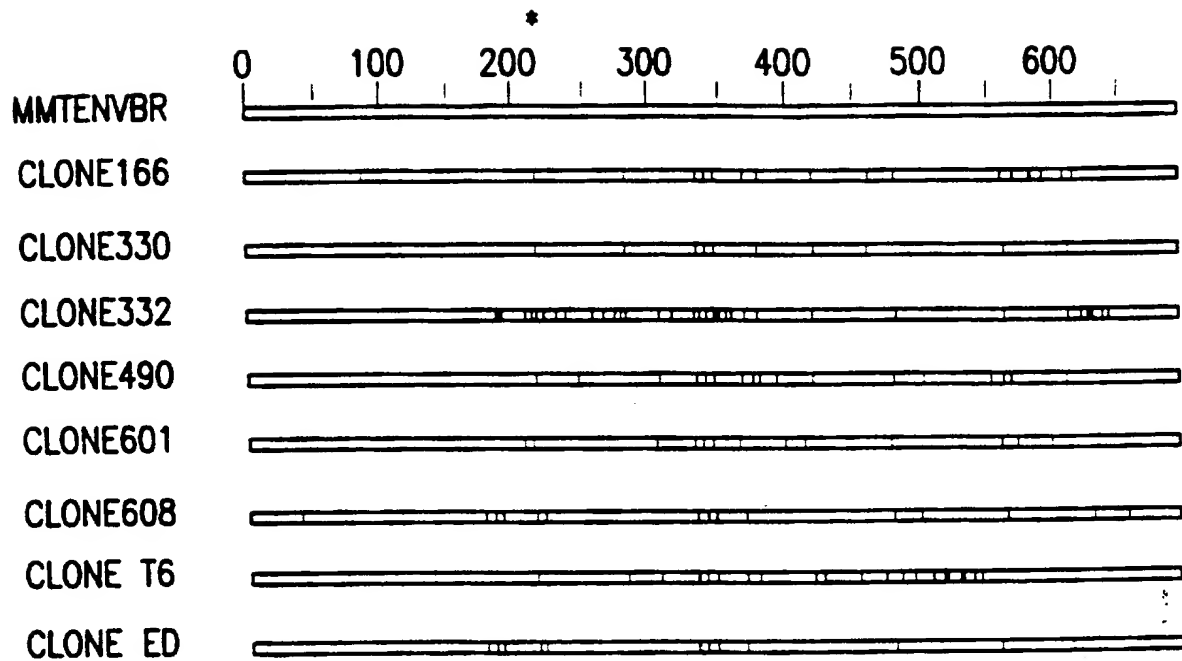


FIG.4

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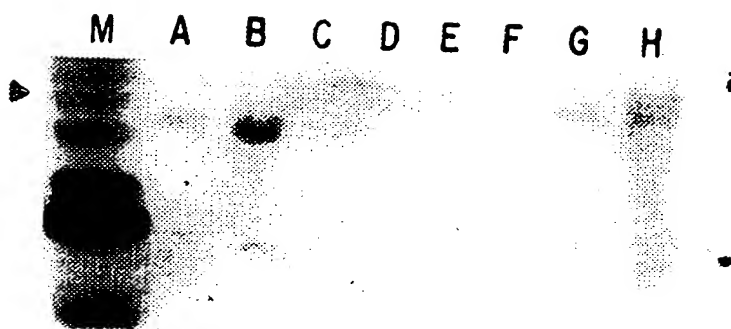


FIG.5

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A B C D E F G H



FIG.6

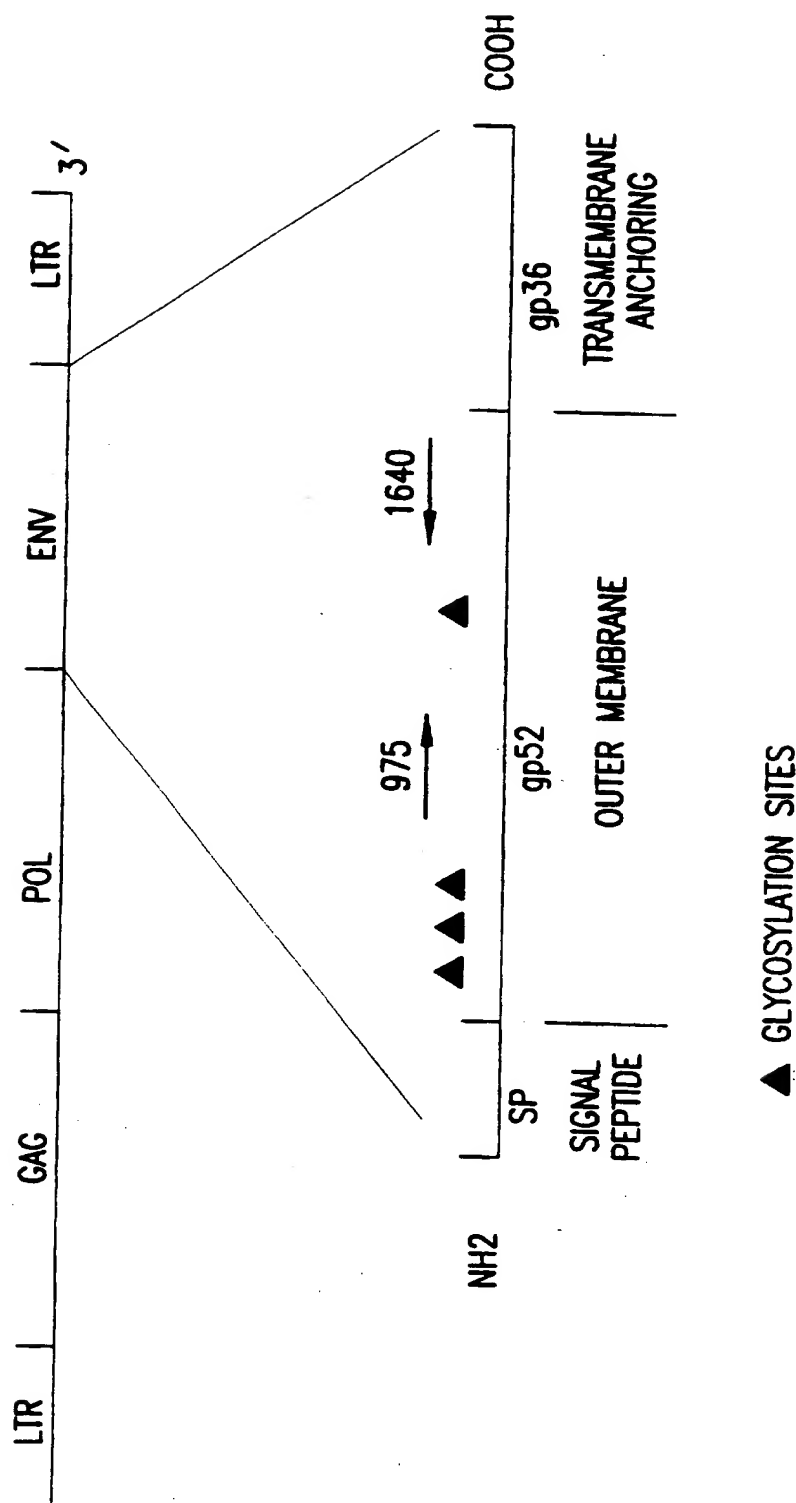


FIG.7

MMTENV 980 | 1000 | 1020 |
[1810] TCTCACTGCGCAGATGCGCT TTAGAAGGAGCGCTTCTCG GAGCGAGACGAGTCTCTCTCC
MS1627.Seq TCTCACTGCGCAGATGCGCT TTAGAAGGAGCGCTTCTCG GAGCGAGACGAGTCTCTCTCC
5 10 15 20 25 30 35 40 45 50 55 60

MMTENV 1040 | 1060 | 1080 |
[1810] TCCAGCGTGGTTGGCTTGGG CCTTCCTCGACCAAGGGGCTG ACTTTTCTCCAAAAGGGG
MS1627.Seq TCCAGCGTGGTTGGCTTGGG CCTTCCTCGACCAAGGGGCTG ACTTTTCTCCAAAAGGGG
65 70 75 80 85 90 95 100 105 110 115 120

MMTENV 1100 | 1120 | 1140 |
[1810] CCTTGGGTTACTTTGGGATT TCTCCCTTCCCTGGGCTAGT GTAGATCACTCAGATCAGAT
MS1627.Seq CCTTGGGTTACTTTGGGATT TCTCCCTTCCCTGGGCTAGT GTAGATCACTCAGATCAGAT
125 130 135 140 145 150 155 160 165 170 175 180

MMTENV 1160 | 1180 | 1200 |
[1810] TAAAAGCAAAAAGCATCTAT TTGAAATTATACTCCCGCA GTCAATAAAGAGGTTTCATCG
MS1627.Seq TAAAAGCAAAAAGCATCTAT TTGAAATTATACTCCCGCT GTCAATAAAGAGGTTTCATCG
185 190 195-200 205 210 215 220 225 230 235 240

MMTENV 1220 | 1240 | 1260 |
[1810] ATGCTATCAAGCAGGATGCG TACAACCTACATGCTTCTCG CAATAATCTCTTAAGGATCC
MS1627.Seq ATGCTATCAAGCAGGATGCG TACAACCTACATGCTTCTCG CAATAATCTCTTAAGGATCC
245 250 255 260 265 270 275 280 285 290 295 300

MMTENV 1280 | 1300 | 1320 |
[1810] CAATCATAGAGATTTTACTG CTCTAGTTCCCATACAGAT TGTTCCTTACTTTCAGCG
MS1627.Seq CAATCATAGAGATTTTACTG CTCTAGTTCCCATACAGAT TGTTCCTTACTTTCAGCG
305 310 315-320 325 330 335 340 345 350 355 360

MMTENV 1340 | 1360 | 1380 |
[1810] TCAAGATATCTTATTCACAA AAGCCAGGATTTCAAGAAC TCAGATC-ATTCCTACATCT
MS1627.Seq TCAAGATATCTTATTCACAA AAGCCAGGATTTCAAGAAC TCAGATC-ATTCCTACATCT
365 370 375 380 385 390 395 400 405 410 415 420

MMTENV 1400 | 1420 | 1440 |
[1810] CTCTCTTACTTACGCTTATG CCAACTATTAGGATTAAGT CAGCTTAATAGATACAGAA
MS1627.Seq CTCTCTTACTTACGCTTATG CCAACTATTAGGATTAAGT CAGCTTAATAGATACAGAA
425 430 435 440 445 450 455 460 465 470 475 480

MMTENV 1460 | 1480 | 1500 |
[1810] AGAGGATCTACTTTTCAAT TTTCTCTTCTTCTTGTAGAT TCACTAATGTTTACAGTCT
MS1627.Seq AGAGGATCTACTTTTCAAT TTTCTCTTCTTCTTGTAGAT TCACTAATGTTTACAGTCT
485 490 495 500 505 510 515 520 525 530 535 540

MMTENV 1520 | 1540 | 1560 |
[1810] TCTGCTTACGACTATGACG CATCAAGTCAAGAGGGGCT CATAGCTGCTCTACCTGTA
MS1627.Seq TCTGCTTACGACTATGACG CATCAAGTCAAGAGGGGCT CATAGCTGCTCTACCTGTA
545 550 555 560 565 570 575 580 585 590 595 600

MMTENV 1580 | 1600 | 1620 | 1640 |
[1810] GATATGCTGATGAGGAGTG GTTGTATGAGTCTGCACTC AAACCTTAGGATTCACACA GAT
MS1627.Seq GATATGCTGATGAGGAGTG GTTGTATGAGTCTGCACTC AAACCTTAGGATTCACACA GAT
605 610 615 620 625 630 635 640 645 650 655 660

FIGURE 8

CGAACAGACACAAACACACGAGAGGTGAATGTTAGGACTGTTGCAAGTTTA
CTCAAAAAACAGCACTCTTTTATATCATGGTTTACATAAGCATTACATAAGA
CTTGGATAAGTTCCAAAAGAACATAGGAGAATAGAACACTCAGAGCTTAGAT
CAAAACATTTGATACCAACCAAGTCAGGAAACCACTTGTCTCACATCCTTG
TTTTAAGAACAGTTTGTGACCCTGAACCTTAAACCTTGGGAACCGCAAN
GTTGGGCTCATAAAGTTATCCATTATAGCTCATGCCAAAATTATCTGCAGA
AATGTGTTCTAATTGTCTAGCCACTGCCCCCTCCCTTGGTATAATGAAAA
CTTTCCCCAACGTTCCACTCCCTAGATAAATAATCATGTACCTGT
TGTTTTATGTCGTCTTTTTCTTCTGAGTTAACACACCAAGGAGGTCTAGC
TCTGGCGAGTCTTTCACGAAAGGGGAGGGATCTGTACAACACTTTATAGCC
GTTGACTGTGACCCACCTATCGAAATTTAAATCGTATCTTCTGTATATGGTA
GCGGGGCGTCTGTTGGTCTGTAGATGTAAGTCCCGGTTGCCACCACCTGTC
TCCTATTTTGACAAGCGTACTCCTCTTCCCCTTTTAGTTCTAGGCCTGAGG
CCCTTAGTCCTTGCACCTGTTCTTCAACTGAGGTGAGCGTCTCTTTCTATT
TCTATTTCCATTTCTAACCTTTGAATTTGAGTAAATATAGTGCTAAAAGACAA
AGATTCATTTCTAACATCATGATTAATAATCGACCTATTGGATTGGTCTTATT
GGTAAAAATATAATTTTAGCAAGCATTCTTATTCTATTCTGAAGGACAAA
GTCGGTGTGGCTTGTAAAGGAANTTGGCTGTGGTCTTGCCCCACGAGGA
AGGTCGAGTCTCCGAATTGTTTAGATTGTAATCTTGACAGAAGAGTTATTA
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AGCCCCAGGCAAAGCAGAGCTATGCCAAGTTTGCAGCAGANAATGAGTATG
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GGAAACAACCCCTTGGCTGCTTCTCTCCTAAGTGTAGGACACTCTCGGGAG
TTCAACCATTTCTGCTGCAGGCGGGCATTTCCCCCTTTTTCTTTTTAAAA
GAAGCACGTTAAGATCTGACTGCACTTGGTCAAGGCTCTTCGCAAAGCACT
GGAAAAATAACGGGGAAAAATCATAAGTACTATGACCAAAAGCAGGGCTCCAA
CTCCTATAAAAAATGAAATATTGTGTTCTAATCCAATGGATTTAAAGCCTTTAC
TCCATTGGCNAAGGANTGANCCAACCCCTGAGGTCCCTGCGTTCAAATTTTT
TTGCTCNTATCCTAATCCAATTGGTAACCCGTTNTTTTTGAAACTCATGTC
TTCAAATGCCCAATAAATGAGCCCTGGTTCTTCCAGCTCTCAGAAGCATT
ATACGGNANAGGTGTGACACAGCATAAAATCATAATTTGCATGACACCTAGT
GGACATTCTGGTCTTTAAGTTTGCCACATCTTGCCCAACTCTAAACTACTT
CTTCTAAAGCATTAAAGTCTAGCTTTCAATTTTAAGTCTATTATTCTTTGTTGAG
ATNAGGCTAATGTAACATTTCTATGAAGATTATTAACAAACGTAGCAGTTTGC
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TTAAAGCAGATATGCCAGAATAATGGCAGCGACGAATCGCTTAGCTCGAAT
TAAATCTGTGGCATAACCTAAAGGTTTGAATGGCAGAATCATCAAACCATGGT
TCATACCAATATCTACAGGTTACAACACATATGGCGGCCCTTGAATATGA
ATCGCTGCATATCCGTNGGCAAAAAATCTAACCATTATTCCTCCTNCCNAAA
AACGGGATTTGAAANTTATNCCCCCTNCCCCNAACCCANACCGAGGTACCC
CATAATGNGGGGGGTATCTANAANAGGGCATAGGGGTAAAGAAAAACGGCA
GAGNGGATCNTTTATGTTTNGGAAATTCNGGGTTTGGGAGAATAAGATTCT
GGAGGCTGCAATTAAGGGAAACATTNTGTATGGGGAATAGAGCAGTAAAA
TCTCTATCATGGGATCTTTAGGGAGAATTTCCAGGAACCAAGTAGGTTT
NAACCCATCNTGCTTCATACCATCGATGAACNTCTTTATTGACAGGGGGAGT
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AAAAACTACCCCTTGGTCAGGGAAGGCGCAAGGCAACCACCGTGGAGGA
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FIG.9

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17877

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 435/6, 5, 91.2, 7.1, 7.2; 536 23.1, 24.3, 24.33; 530/388.1, 300

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 5, 91.2, 7.1, 7.2; 536 23.1, 24.3, 24.33; 530/388.1, 300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	REDMOND et al. Sequence and expression of the mouse mammary tumour virus env gene. The EMBO Journal. 1983, Volume 2, Number 1, pages 125-131. See entire document.	1-20
A	FAFF et al., Retrovirus-like particles from the human T47D cell lines are related to mouse mammary tumour virus and are of human endogenous origin. Journal of General Virology. 21 May 1992, Volume 73, pages 1087-1097. See abstract.	1-20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance		
E earlier document published on or after the international filing date	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed	*Z*	document member of the same patent family

Date of the actual completion of the international search

04 FEBRUARY 1997

Date of mailing of the international search report

18 MAR 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

DIANNE REES

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17877

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CREPIN et al. Sequences Related to Mouse Mammary Tumor Virus Genome in Tumor Cells and Lymphocytes from Patients with Breast Cancer. Biochemical and Biophysical Research Communications. 13 January 1984, Volume 118, Number 1, pages 324-331. See entire document.	1-20
A	MESA-TEJADA et al. Detection in human breast carcinomas of an antigen immunologically related to a group-specific antigen of mouse mammary tumor virus. Proceedings of the National Academy of Sciences, USA. March 1978, Volume 75, Number 3, pages 1529-1533.	1-20*

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17877

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C12Q 1/68, 1/70; C12P 19/34; C07H 21/02, 21/04; G01N 33/53; C07K 15/28; 5/00

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, BIOTECHABS, BIOTECHDS, CABA, CAPLUS, CANCERLIT, DGENE, DRUGU, EMBASES, MEDLINE, USPATFULL, TOXLIT, TOXLINE, JAPIO, WPIDS
search terms: MMTV, mouse mammary tumor virus, PCR, hybridization, antibodies, immunoassays, Westerns, searched SEQ. ID. Nos.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68, 1/70, C12P 19/34, C07H 21/02, 21/04, G01N 33/53, C07K 15/28, 5/00	A1	(11) International Publication Number: WO 97/17470 (43) International Publication Date: 15 May 1997 (15.05.97)
(21) International Application Number: PCT/US96/17877 (22) International Filing Date: 8 November 1996 (08.11.96) (30) Priority Data: 08/555,394 9 November 1995 (09.11.95) US (71)(72) Applicant and Inventor: HOLLAND, James, F. [US/US]; 31 Mamaroneck Road, Scarsdale, NY 10583 (US). (72) Inventor: POGO, Beatriz, G., T.; 237 Nyac Avenue, Pelham, NY 10803 (US). (74) Agents: CLARK, Richard, S. et al.; Brumbaugh, Graves, Donohue & Raymond, 30 Rockefeller Plaza, New York, NY 10112 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: DETECTION OF MAMMARY TUMOR VIRUS-LIKE SEQUENCES IN HUMAN BREAST CANCER (57) Abstract The present invention relates to materials and methods for diagnosing breast cancer in humans. It is based, at least in part, on the discovery that a substantial percentage of human breast cancer tissue samples contained nucleic acid sequences corresponding to a portion of the mouse mammary tumor virus <u>env</u> gene. In contrast, such sequences were absent in almost all other human tissues tested.		

* (Referred to in PCT Gazette No. 31/1997, Section II)

** (Referred to in PCT Gazette No. 49/1997, Section II)

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Description

Detection Of Mammary Tumor Virus-Like Sequences In Human Breast Cancer

Cross-Reference to Related Application

This application is a continuation-in-part application of U.S. Serial No. 08/555,394, filed November 9, 1995.

Statement Regarding Federally Sponsored Research

- 5 This invention was made with funds from the U.S. government, which has certain rights in the invention.

Introduction

- 10 The present invention relates to materials and methods for diagnosing breast cancer in humans. It is based, at least in part, on the discovery that a substantial percentage of human breast cancer tissue samples contained nucleic acid sequences corresponding to a portion of the mouse mammary tumor virus env gene. In contrast, such sequences were absent in almost all
15 other human tissues tested.

Background of the Invention

- 20 A large body of information has accumulated about the molecular biology of MMTV (reviewed in Slagle, B.L. et al., 1987, in "Cellular and Molecular Biology of Mammary Cancer", Kidwell et al., eds., Plenum Press, NY. pp 275-306). Mouse mammary tumor virus (MMTV) is associated with a high incidence of breast cancer in certain strains of mice (over 90% among females), and has been regarded as a potential model for human
25 disease.

 The MMTV virus does not carry a transforming oncogene, but rather acts as an insertional mutagen with several proviral insertion loci designated int-1

or wnt-1 (Nusse R. et al., 1982, Cell 31:99-109) int-2
(Peters, G. et al., 1983, Cell 33:369-377) int-3
(Gallahan, D. et al., 1987, J. Virol. 61:218-220) int-4
(Roelink, H. et al., 1990, Proc. Natl. acad. Sci. USA
5 87:4519-4523) and int-5 (Morris, V.L., et al. 1991,
Oncogene Research 6:53-63), which encode for growth
factors or other related proteins. These genes are not
expressed in normal mammary tissue but become activated
after integration of MMTV provirus into the adjacent
10 chromosomal DNA.

The human homolog of the int-2 locus has been
located on chromosome 11 (Casey, G. et al., 1986,
Mol. Cell Biol. 6:502-510) and has been found amplified
(in 15% of the breast cancers) and also expressed
15 (Lidereau, R. et al., 1988, Oncogene Res 2:285-291;
Zhou, D.J. et al., 1988, Oncogene 2:279-282; Liscia,
D.S. et al., 1989, Oncogene 4:1219-1224; Meyers, S.L.
et al., 1990, Cancer Res 50:5911-5918). It may be
significant that in tumors from Parsi women, who have a
20 high incidence of breast tumors, the int-2 locus is
amplified in 50% of the cases (Barnabas-Sohi, N. et
al., 1993, Breast Dis. 6:13-26). The amplification of
int-2 and other genes in 11q13 is indicative of poor
prognosis (Schuwring, E. et al., 1992, Cancer Research
25 52:5229-5234; Champeme, M-H, et al., 1995, Genes,
Chromosomes and Cancer 12:128-133). Both mouse and
human int-2 have been sequenced (Moore, R. et al.,
1986, EMBO J 5:919-924). The gene encodes a protein of
about 27 kilodaltons (KD) which shows homology to both
30 basic and acidic fibroblast growth factors (Dickson, C.
et al. 1987, Nature (London) 326:833).

However, efforts to demonstrate the presence of
viruses in human breast cancer through search for viral
particles, immunological cross-reactivity, or sequence
35 homology have yielded contradictory results. Detect-
able MMTV env gene-related antigenic reactivity
has been found in tissue sections of breast cancer

(Mesa-Tejada et al., 1978, Proc. Natl. Acad. Sci. USA 75:1529-1533; Levine, P. et al., 1980, Proc. Am. Assoc. Cancer Res. 21:170; Lloyd, R. et al., 1983, Cancer 51:654-661), breast cancer cells in culture (Litvinov, S.V. and Golovkina, T.V., 1989, Acta Virologica 33:137-142), human milk (Zotter S. et al., 1980, Eur. J. Cancer 16:455-467) in sera of patients (Day, N.K. et al., 1981, Proc. Natl. Acad. Sci. USA 78:2483-2487), in cyst fluid (Witkin, S.S. et al., 1981, J. Clin. Invest. 67:216-222) and in particles produced by a human breast carcinoma cell line (Keydar, I. et al., 1984, Proc. Natl. Acad. Sci. USA 81:4188-4192). Sequence homology to MMTV has been found in human DNA under low stringency conditions of hybridization (Callahan, R. et al., 1982, Proc. Natl. Acad. Sci. USA 79:5503-5507) and RNA related to MMTV has been detected in human breast cancer cells (Axel, R. et al., 1972, Nature 235:32-36). The presence of MMTV related sequences in lymphocytes from patients with breast cancer has been reported (Crepin, M. et al., 1984, Biochem. Biophys. Res. Comm. 118:324-331), as well as detection of reverse transcriptase (RT) activity in their monocytes (Al-Sumidaie, A.M. et al., 1988, Lancet 1:5-8). May and Westley (May and Westley, 1989, Cancer Research 49:3879-3883) have reported the presence of MMTV-like sequences arranged as tandem repeats only in DNA from breast cancer cells.

These results have been difficult to interpret, and theories linking MMTV or a related virus with human breast cancer have fallen out of favor, in view of the relatively recent discovery of human endogenous retroviral sequences ("HERs"; Westley, B. et al., 1986, J. Virol. 60:743-749; Ono, M. et al., 1986, J. Virol. 60:589-598; Faff, O. et al., 1992, J. Gen. Virology 73:1087-1097). Data which could be interpreted to demonstrate the presence of MMTV-related sequences could be more readily explained by endogenous human

retroviral sequences. Adding further confusion to the picture, env-gene related antigenicity has been detected in epitopes of human proteins (Hareuveni, M. et al., 1990, Int. J. Cancer 46:1134-1135).

5 Brief Summary of the Invention

The present invention relates to methods for diagnosing breast cancer in humans in which the presence of mouse mammary tumor virus env gene-like sequences bears a positive correlation to the existence
10 of malignant breast disease. It is based, at least in part, on the discovery that 38 to 40 percent of human breast cancer tissue samples tested contained gene sequences homologous to the mouse mammary tumor virus env gene that are substantially absent from other human
15 tumors and tissues. The invention also relates to methods for diagnosing breast cancer in humans in which the presence of retrovirus proviral fragments substantially homologous to the env gene and/or 3' LTR sequence of MMTV are detected. The molecular probes
20 used in these experiments were designed to avoid cross-hybridization with endogenous human retroviral sequences. The present invention further provides for compositions of molecular probes which may be utilized in such diagnostic methods.

25 Brief Description of the Figures

FIGURE 1: Amplification of 660 bp of MMTV-like env gene. DNA was extracted from frozen tissues. PCR was performed using primers 1 and 3. A: 2% agarose gel electrophoresis. B: Southern blot hybridization
30 using 5',³²P-end-labeled probe 2. Lanes 1 and 3: breast cancer; lanes 2 and 4: normal breast; lane 5: control reaction (no DNA); lane E: MMTV env gene. M: molecular weight marker. Arrow indicates 510 bp band.

FIGURE 2: Nested PCR. A: 2% agarose gel electrophoresis. 1: Amplification of 686 bp of MMTV-like env
35

gene sequences using primers 1 and 4 and the product of reaction A 1 as template. 2: Amplification of 250 bp of MMTV-like env gene sequences using primers 2 and 3. B, 1 and 2: Southern blot hybridization of the amplified products using probe 5'-³²P end-labeled probe 2a.

FIGURE 3: Amplification of 250 bp of MMTV-like env gene. DNA was extracted from paraffin-embedded tissue sections. PCR was performed using primers 2 and 3. A: 2% agarose gel electrophoresis. B: Southern blot hybridization using 5'-³²P-labeled probe 2a. Lane 1: normal breast; lanes 2 to 5: breast cancer; lane E: MMTV env gene. M: molecular weight marker. Arrow indicates 298 bp band.

FIGURE 4: Nucleotide sequence of the cloned MMTV env gene-like sequences as compared to the env sequences of the GR and BR6 strains of MMTV using the GCG program. *:potential glycosylation site, |:mismatch to MMTV.

FIGURE 5: Southern blot hybridization of genomic DNA. DNA was extracted from frozen tissues or cell lines, digested with EcoRI and transferred to nitrocellulose paper. Hybridization with ³²P-labeled clone 166. DNA from A, B, and G: env gene positive breast cancer; C and D: env negative breast cancer; E and F: normal breast; H: MCF-7 cells. M: molecular weight marker, Arrow indicates 9kb band.

FIGURE 6: Southern blot hybridization of genomic DNA. Experimental conditions as in Fig. 5. DNA from A and B: env negative breast cancer; C and D: env positive breast cancer; E: molecular weight marker (non-labelled); F. to H: normal breast. Arrow indicates position of 9 kb marker.

FIGURE 7: Map of MMTV.

FIGURE 8: Comparison of the nucleic acid sequence of mouse mammary tumor env gene ("MMTENV"), showing residues 976-1640, with the nucleic acid sequence of a

representative 660 bp sequence obtained by PCR reaction of DNA from human breast cancer tissue ("MS1627").

FIGURE 9: Sequence of an about 2.6 kb MMTV-like fragment detected in a human breast carcinoma.

5 Detailed Description of the Invention

The present invention relates to methods and compositions for diagnosing breast cancer in humans.

The present invention provides for compositions comprising an isolated and purified nucleic acid molecule which (i) hybridizes to a gene of mouse mammary tumor virus; (ii) is present in at least 20 percent of DNA samples prepared from breast cancer tissue of different human subjects; and (iii) is present in less than 5 percent of DNA samples prepared from tissues other than breast cancer tissue from different human subjects. A "gene of mouse mammary tumor virus" includes, but is not limited to, the gag, pol, and env genes and the 5' LTR and 3' LTR sequences of MMTV. In preferred embodiments of the invention, the mouse mammary tumor virus (hereafter "MMTV") gene is the env gene and/or the 3' LTR sequence. The term "hybridize" is used to refer to routine DNA-DNA or DNA-RNA hybridization techniques under what would be regarded, by the skilled artisan, as stringent hybridization conditions. The phrase "is present" indicates that a native form of the molecule, in an unpurified state (for example, as part of chromosomal DNA), may be detected by a standard laboratory technique, such as Southern blot or polymerase chain reaction (PCR). To be "present", the molecule may be detectable by one technique but not others. To be present in "less than 5 percent of DNA samples prepared from tissues other than breast cancer tissue from different human subjects", all non-breast cancer tissue samples are considered together, but the total number of samples must be large enough to give the 5 percent

value statistical significance that would be reasonable to the skilled artisan.

In order to identify such a nucleic acid molecule, the sequence of MMTV may be compared, using a computer database, to known human DNA sequences, and portions of MMTV which are less than or equal to 25 percent homologous to a human sequence may be selected for further study. The term "homologous", as used herein, refers to the presence of identical residues; for example, a first sequence is considered 25 percent homologous to a second sequence if it shares 25 percent of the residues of the first sequence. Since there is relatively greater likelihood that MMTV may bear similarity to human retroviral-like sequences, it may be preferable to evaluate whether a particular MMTV nucleic acid sequence is homologous to such sequences, for example, as endogenous human retrovirus sequences. A prototype of such viruses is HERV-K10 (Ono, M. et al., 1986, J. Virol. 60:589-598).

Once an MMTV gene sequence which is less than or equal to 25 percent homologous to a human DNA sequence, such as a human endogenous retroviral sequence, is identified, the presence of nucleic acid molecules having the MMTV gene sequence in human breast cancer tissues and other tissues may be evaluated. Such evaluations may be performed either by Southern blot techniques, or, preferably, by polymerase chain reaction (PCR) techniques, which are more sensitive. In such a way, MMTV gene sequences which (i) hybridize to at least 20 percent of DNA samples prepared from breast cancer tissue of different human subjects and (ii) hybridize to less than 5 percent of DNA samples prepared from human tissues other than breast cancer tissues may be identified. A nucleic acid molecule having a MMTV gene sequence which satisfies these requirements may then be used in diagnostic methods which detect the presence of such sequence in human

breast tissue by standard techniques, including PCR techniques which assay for the presence of the molecule, but also, where appropriate, Southern blot, Northern blot, or Western blot techniques, to name but a few.

In preferred embodiments, the present invention relates to a portion of MMTV localized between MMTV env gene sequences 976 and 1640 (Majors, I.E. and Varmus, H.E., 1983, J. Virol. 47:495-504; see Fig. 7). This about 660 bp sequence (hereafter, "the 660 bp sequence") has been found to exhibit low (16 percent) homology to the prototype human endogenous retrovirus HERV-K10, using the IBI/Pustell Sequence Analysis Program, and has also been shown to be present in 121 (38.5%) of 314 unselected breast cancer tissue samples, in cultured breast cancer cells, in 2 of 29 breast fibroadenomas (6.9%) and in 2 of 107 breast specimens from reduction mammoplasties (1.8%). The sequence was not found in normal tissues including breast, lymphocytes from breast cancer patients nor in other human cancers or cell lines (see example section, infra). Similarly, an about 250 bp sequence (hereafter "the 250 bp sequence"), between positions 1388 and 1640 in the env gene, and therefore falling within the 660 bp sequence, was detected in 60 (39.7%) of 151 breast cancer, and in one of 27 normal breast samples assayed from paraffin-embedded sections. Cloning and sequencing of the 660 bp and 250 bp sequences demonstrated that they are 95-99% homologous to MMTV env gene, but not to the known human endogenous retroviruses ("HERs") nor to other viral or human genes (<18%).

In another preferred embodiment, the present invention relates to a nucleic acid molecule which corresponds to a retroviral genomic fragment which has substantial homology to 3' LTR and/or env gene of the MMTV genome, and is found in a substantial percentage of breast cancer samples. By substantial percentage is

meant at least 20% of tested breast cancer samples. Such a sequence is preferably comprised of the 3' LTR region and all or part of the env gene, although it may include more sequences of a retroviral genome. Most preferably, the sequence is at least comprised of an about 2.6 kb fragment which comprises the 1,228 base pair (bp) sequence of the 3' LTR sequence and 1,336 bp of the env gene sequence of MMTV (Fig. 9) (SEQ ID NO:20). When compared with the two strains of MMTV C3H and BR6, the sequence homology was 90.8% and 90.7%, respectively. When compared with the endogenous retroviral sequences (HUMERKA), sequence homology was only 58% in 36 bp and 71% in 74 bp.

Retrovirus proviral sequences can be detected by PCR technology using primers derived from the MMTV genome. Such primers include primer 5L, containing the nucleotides 7376-7395 of the MMTV BR6 genome (5'-3': CCAGATCGCCTTTAAGAAGG) (SEQ ID NO:11) and primer LTR3, containing nucleotides 9918-9927 of the MMTV BR6 genome (5'-3': CGAACAGACACAAAGCGACG) (SEQ ID NO:19). Other primers which correspond to or are homologous to MMTV sequences can be used as primers. Nucleotide fragments which correspond to or are homologous to the retroviral sequences isolated from the breast cancer samples can also be used to amplify additional retroviral fragments from the samples. Long PCR techniques can be used to amplify longer stretches of a proviral sequence.

The present invention provides for compositions comprising an isolated and purified nucleic acid molecule which hybridizes to the about 2.6 kb retroviral fragment shown in Fig. 9 under stringent conditions or is at least 90 percent homologous to said fragment using the MacVector homology determining program which may be used to diagnose breast cancer in a subject, using methods which include PCR and Southern blot methods.

Nucleic acids having the 660 bp sequence, the 250 bp sequence, or all or part of the about 2.6 kb sequence, may therefore be used, according to the invention, to diagnose breast cancer in a subject, using methods which include PCR and Southern blot methods. Where PCR methods are used, primers such as those listed in Table 1, below, may be utilized.

The present invention provides for compositions comprising essentially purified and isolated nucleic acid having the 660 bp sequence or the 250 bp sequence or an at least five bp, and preferably greater than or equal to ten bp, subsequence thereof. In order to maintain the desired specificity, such nucleic acid molecules may preferably contain sequence falling within the 660 bp sequence, but preferably do not contain sequences from other portions of the MMTV genome, which may, undesirably, hybridize to human sequences which are not breast cancer specific, such as HERs. Accordingly, the present invention provides for compositions wherein the isolated and purified nucleic acid molecule comprises at least a portion having a nucleic acid sequence which hybridizes to a region of the mouse mammary tumor virus env gene between residues 976 and 1640, or between residues 1388 and 1640, and wherein the isolated and purified nucleic acid molecule does not hybridize to any other region of the MMTV genome.

The 660 bp sequence, in various embodiments, may have a number of nucleotide sequences. For example, in one embodiment, the 660 bp sequence may have a sequence as set forth in Fig. 8 and designated "MMTENV-like sequence" (SEQ ID NO:17), which depicts the MMTV env sequence between residues 976 and 1640. In a second series of embodiments, the 660 bp sequence may have a sequence as set forth in Fig. 8 and designated "MS1627" (SEQ ID NO:18), which depicts a predominant sequence for the 660 bp sequence as it has been defined by

sequencing analysis of the products of PCR reactions using DNA from human breast cancer tissues. In still further embodiments, the 660 bp sequence may have various other nucleotide sequences obtained by
5 sequencing the results of PCR reactions to detect the presence of 660 bp sequence in human breast cancer tissues.

In related embodiments, the present invention provides for compositions comprising PCR primers
10 that may be used to detect the presence of the forementioned molecules or other MMTV-like sequences. For example, the compositions may comprise one or more of the following primer molecules (5' - 3'):
CCTCACTGCCAGATC (SEQ ID NO:1); GGGAATTCCTCACTGCCAGATC
15 (SEQ ID NO:2); CCTCACTGCCAGATCGCCT (SEQ ID NO:3);
TACATCTGCCTGTGTTAC (SEQ ID NO:4); CCTACATCTGCCTGTGTTAC
(SEQ ID NO:5); CCGCCATACGTGCTG (SEQ ID NO:6);
ATCTGTGGCATAACCT (SEQ ID NO:7); GGGAATTCATCTGTGGCATAACCT
(SEQ ID NO:8); ATCTGTGGCATAACCTAAAGG (SEQ ID NO:9);
20 GAATCGCTTGGCTCG (SEQ ID NO:10); CCAGATCGCCTTTAAGAAGG
(SEQ ID NO:11); TACAGGTAGCAGCACGTATG (SEQ ID NO:12);
CGAACAGACACAAACACACG (SEQ ID NO:19).

The use of such compositions and molecules in PCR and Southern blot techniques is illustrated in the non-
25 limiting examples set forth below. The correlation between the presence of the MMTV-related nucleic acid molecules described above and breast cancer allows such molecules and compositions to be utilized in the diagnosis of breast cancer. Accordingly, the present
30 invention provides for a method of diagnosing breast cancer, wherein the detection of such nucleic acid molecules bears a positive correlation to the existence of breast cancer in a human. The results of such
evaluation, together with additional clinical symptoms,
35 signs, and laboratory test values, may be used to formulate the complete diagnosis of the patient.

In further related embodiments, the present invention provides for an essentially purified peptide encoded by a nucleic acid molecule which (i) hybridizes to a gene of MMTV; (ii) is present in at least
5 20 percent of DNA samples prepared from breast cancer tissue of different human subjects; and (iii) is present in less than 5 percent of DNA samples prepared from tissues other than breast cancer tissue from different human subjects. In preferred embodiments, the
10 MMTV gene is the env gene.

Such peptides may be used in the diagnosis of breast cancer. Accordingly, the present invention provides for a method of diagnosing breast cancer in a human subject, comprising detecting the presence of
15 a peptide encoded by a nucleic acid molecule which (i) hybridizes to the env gene of a mouse mammary tumor virus; (ii) is present in at least 20 percent of DNA samples prepared from breast cancer tissue of different human subjects; and (iii) is present in less than
20 5 percent of DNA samples prepared from tissues other than breast cancer tissue from different human subjects.

The present invention also provides for antibodies (including monoclonal and polyclonal) antibodies which
25 specifically bind to such peptides. Such antibodies may be used in methods of diagnosing breast cancer, for example, but not by way of limitation, by Western blot, immunofluorescent techniques, and so forth.

In nonlimiting embodiments of the invention, the
30 skilled artisan may evaluate MMTV-like nucleic acid molecules for regions which would be considered likely to encode immunogenic peptides (using, for example, hydropathy plots). Such peptides may then be sequenced and used to produce antibodies that may be employed in
35 diagnostic methods as set forth above.

For example, certain peptides encoded by portions of the 660 bp sequence have been synthesized. These

peptides, which have the sequences LKRPGFQEHMI (SEQ ID NO:13) and GLPHLIDIEKRG (SEQ ID NO:14), have been used to produce antibodies in rabbits, and the resulting antisera have successfully identified breast cancer cells positive for MMTV env-like sequences by PCR assay. Other peptides encoded by 660 bp sequence which may be useful according to the invention include TNCLDSSAYDTA (SEQ ID NO:15) and DIGDEPWFDD (SEQ ID NO:16).

6. Example: The Detection of Mouse Mammary Tumor Virus Env Gene-Like Sequences in Human Breast Cancer Cells and Tissues

6.1. Materials and Methods

DNA from breast cancer tissue and other human cancer tissues, human placentas, normal human tissues including breast, and from several human cell lines (including eight breast cancer cell lines), and two normal breast cell lines was extracted following the procedure of Delli Bovi et al. (1986, Cancer Res. 46:6333-6338). The DNA was resuspended in a solution containing 0.05 M Tris HCl buffer, pH 7.8, and 0.1 mM EDTA, and the amount of DNA recovered was determined by microfluorometry using Hoechst 33258 dye (Cesarone, C. et al., 1979, Anal Biochem 100:188-197). Plasmids containing the cloned genes of MMTV were obtained from the ATCC, propagated in Escherichia coli cultures and purified using anion-exchange minicolumns (Qiagen) or by precipitation with polyethylene glycol (Sambrook J., et al., 1989, in "Molecular Cloning/A Laboratory Manual", Cold Spring Harbor). Oligonucleotide primers were synthesized at the core facilities of the Brookdale Molecular Biology Center at Mount Sinai School of Medicine.

Polymerase chain reaction (PCR) was performed using Taq polymerase following the conditions recommended by the manufacturer (Perkin Elmer Cetus)

with regard to buffer, Mg^{2+} and nucleotide concentrations. Thermocycling was performed in a DNA cycler by denaturation at 94° C for 3 min. followed by either 35 or 50 cycles of 94° C for 1.5 min., 50° C for 2 min. and 72° C for 3 min. The ability of the PCR to amplify the selected regions of the MMTV env gene was tested by using as positive templates the cloned MMTV env gene and the genomic DNA of the MCF-7 cell line, since it was shown to express gp52 immunological determinants (Yang, N.S., et al., 1975, J. Natl. Cancer Inst. 61:1205-1208). Optimal Mg^{2+} , primer concentrations and requirements for the different cycling temperatures were determined with these templates. The master mix as recommended by the manufacturer was used. To detect possible contamination of the master mix components, a reaction without template was routinely tested. γ DNA and control primers provided by the manufacturer were used as control for polymerase activity. As an internal control, amplification of a 120 bp sequence estrogen receptor gene was assayed using primers designed and generously provided by Dr. Beth Schachter, (Mount Sinai School of Medicine, N.Y.). In addition, primers for actin 5 gene amplification were also used.

The product of the PCR was analyzed by electrophoresis in a 2% agarose gel. A 1 kb DNA ladder (Gibco BRL) was used to identify the size of the PCR product. To determine if the amplified sequences of the middle region of the 660 bp faithfully reproduced the sequences of the env gene of MMTV, an 18-mer sequence within the env gene was used as a probe for the 660 bp amplified sequence. The 18-mer probe was 5' end-labeled with ^{32}P -ATP using T4 polynucleotide kinase and purified by the NENSORB nucleic acid purification cartridge (NEN). Southern blot hybridization was performed using the conditions described by (Saiki et al., 1985, Science 230:1350-1354).

The product of the PCR (660 bp or 250 bp) was cloned directly from the reaction mixture into the TA cloning vector (Invitrogen) using the TA cloning kit and following the conditions recommended by the
5 supplier. Direct cloning of the fragment isolated from the gel, was also performed. Plasmid DNA was purified by CsCl density gradient centrifugation or by precipitation with polyethylene glycol (Sambrook et al., 1989, in "Molecular Cloning/A Laboratory
10 Manual", Cold Spring Harbor), restricted with HindIII and EcoRI, electrophoresed in 2% agarose gels and transferred to nitrocellulose filters. Southern blot hybridization was carried out using a 5'-terminal labeled internal probe as described above. Cloning
15 procedures were performed in laboratories totally separate from those where PCR was carried out. Automated DNA sequencing (using Applied Technology Sequencer Model 373A) was performed in the Brookdale Molecular Biology Center. Sequence homology was
20 determined using the IBI MacVector GenBank and GCG Programs.

To prevent contamination of the samples, processing of human tissues was performed in a laminar flow hood. DNA extractions were done in a chemical hood
25 located in a different room from that where PCR was performed. PCR assays were assembled in a biological hood provided with ultraviolet light. Aerosol resistant tips and dedicated positive-displacement pipettes were used throughout. All equipment used for
30 PCR (microcentrifuge, electrophoresis apparatus, pipettors) was cleaned each time with 10% sodium hypochlorite to assure DNA decontamination (Prince and Andrus, 1992, Biotechniques 12:358-36). After the initial experiments were performed, the plasmid con-
35 taining the MMTV env gene was frozen and never used again, to avoid contamination. However, to detect plasmid contamination from our own env gene clones,

primers were designed to amplify plasmid sequences. All the authentic MMTV env positive samples were then tested and found negative for plasmid contamination.

Southern blotting and hybridization were performed as described (Southern, E.M., 1975, J. Mol. Biol. 98:503-517), using the 660 bp cloned sequences labeled by the random primer procedure (Feinberg, A.P., et al., 1983, Anal. Biochem. 132:6-13). Prehybridization and hybridization were performed in a solution containing 6 x SSPE, 5% Denhardt's, 0.5% SDS, 50% formamide, 100 µg/ml denaturated salmon testis DNA, incubated for 18 hrs at 42°C, followed by washings with 2 x SSC and 0.5% SDS at room temperature and at 37°C and finally in 0.1 x SSC with 0.5% SDS at 68°C for 30 min (Sambrook et al., 1989, in "Molecular Cloning/A Laboratory Manual", Cold Spring Harbor). For paraffin-embedded tissue sections the conditions described by Wright and Manos (1990, in "PCR Protocols", Innis et al., eds., Academic Press, pp. 153-158) were followed using primers designed to detect a 250 bp sequence.

6.2. Results

6.2.1. Selection of Specific MMTV Env Gene Sequences

A computer search for MMTV env gene homologous sequences was first performed, since sequence homology between the human endogenous retroviral sequences and MMTV had been described. The prototype of this group of human endogenous retroviruses is HERV-K10 (Ono, M. et al., 1986, J. Virol. 60:589-598). The sequences of the env gene of MMTV (Majors, I.E. and Varmus, H.E., 1983, J Virol 47:495-504) were aligned with sequences of the env gene of the human endogenous retrovirus HERV-K10 (Ono, M. et al., 1986, J. Virol. 60:589-598), using the IBI/Pustell Sequence Analysis Program. A region of 660 bp of low homology (16%) was localized between MMTV env gene sequences 976 and 1640 (Majors, I.E. and Varmus, H.E., 1983, J Virol 47:495-504). This

internal domain of the outer membrane of the env gene has only one glycosylation site and is highly conserved between strains. Two primers comprising 15 bp sequences at positions 976-990 (primer 1) and 1626-1640 (primer 3) were first synthesized. Later longer primers were synthesized (1N and 3N). An 18-mer sequence in the middle of the 660 bp MMTV env region (1388-1405) (primer 2) was used as a probe to identify the 660 bp sequence. A second oligomer probe was synthesized comprising the sequence 1554 to 1568 (primer 2a) to be used for hybridization when a sequence of around 250 bp (between positions 1388 and 1640) was amplified. For nested PCR reactions (Mullis, K.B. and Faloona, F.A., 1987, Meth Enzymol 155:335-350), another primer comprising sequences 1647 to 1661 (primer 4) was synthesized to be used with primer 1 in the first reaction and primers 2 and 3 in the second. Modified primers with GC clamps and extra sequences were also synthesized and used in the PCR (primers 1a and 3a). Another set of primers comprising sequences 974 to 1003 (5L) and 1558 to 1577 (3L) were subsequently developed because their T_m's matched and provided better amplification than the original primers. The sequences are represented in Table 1. All of them were productive in amplification reactions.

Table 1. Primer and probe sequences and location in mouse mammary tumor virus env gene

	Designation	Sequence (5'-3')	Location
5	1	CCTCACTGCCAGATC	976-990
	1a	GGGAATTCCTCACTGCCAGATC	976-990
	1N	CCTCACTGCCAGATCGCCT	976-993
	2	TACATCTGCCTGTGTTAC	1388-1405
10	2N	CCTACATCTGCCTGTGTTAC	1386-1405
	2a	CCGCCATACGTGCTG	1554-1568
	3	ATCTGTGGCATAACCT	1640-1626
	3a	GGGAATTCATCTGTGGCATAACCT	1640-1626
	3N	ATCTGTGGCATAACCTAAAGG	1640-1621
15	4	GAATCGCTTGGCTCG	1661-1647
	5L	CCAGATCGCCTTTAAGAAGG	984-1003
	3L	TACAGGTAGCAGCACGTATG	1558-1577

6.2.2. Detection of MMTV-Like Env Gene Sequences in Human Breast Tumor DNA

20 PCR was performed on DNA extracted from breast cancer tissues, normal breast tissues and from the plasmid containing the env gene of MMTV, using primers 1 and 3. Photographs of the ethidium bromide stained gels of the PCR product reveal the presence of an

25 approximately 660 bp sequence in some of the tumors, (Fig. 1A, lanes 1 and 3) but not in the normal tissue samples (Fig. 1A, lanes 2 and 4). As a positive control the MMTV env gene was also amplified (Fig. 1A, lane E). Similar results were obtained with modified

30 primers 1a, 3a, 3L and 5L. Southern blot hybridization of the gel with ³²P-labeled 18-mer oligonucleotide (primer 2) indicated that this internal sequence was present in the amplified material (Fig. 1B) and that the bands in the gel were not artifactual.

35 Our initial effort was to analyze a representative sample of breast cancer specimens as well as normal

tissues and other tumors. To date 343 breast tumors have been processed, DNA extracted and PCR performed. Of these 343 tumors, 314 were carcinomas and 29 were fibroadenomas. Amplification of sequences of 660 bp was observed in 121 of the carcinomas (38.5%) and in 2 of the 29 fibroadenomas (6.9%). These sequences were confirmed to be MMTV env gene-like sequences by hybridization with the labeled specific probe containing the internal sequences. These sequences were not detected in the DNAs extracted from 20 normal organs, 23 cancers from other organs and 26 samples of blood lymphocytes including 7 from breast cancer patients whose breast specimens were positive. From 107 samples of normal breast obtained from reduction mammoplasties, 2 were positive (1.8%). In addition to DNA from lymphocytes from seven positive patients, DNA from their normal breast tissue of the operated breast was tested in 4 cases. All were negative (Table 2). Finally, DNA of the MCF-7, and ED (a cell line developed in our laboratory from the pleural effusion of a patient with an env -positive breast tumor) breast cancer cell lines were shown to contain the 660 bp MMTV env gene-like sequences (Table 3), while four other breast cancer cell lines were positive only for the 250 bp sequence (T47-D, BT-474, BT-20 and MDA-MB-231).

**Tabl 2. Detection of MMTV env gene-like
s quences in human DNA extract d
from fresh or frozen tissues**

5				
	Sample	Number	MMTV <u>env</u> gene sequences	% Positive
	Breast Carcinomas	314	121	38.5%
	Breast Fibroadenomas	29	2	6.9%
10	Normal Breasts	107	2	1.8%
	*Normal Breasts	4	negative	
	Tumors other than breast	23	negative	
	Normal tissues	20	negative	
15	Lymphocytes	26	negative	
	**Lymphocytes	7	negative	
	* Histologically normal tissue from same breast as positive cancer.			
20	** Lymphocytes from breast cancer patients who were positive for MMTV <u>env</u> gene sequences in the tumor.			

Table 3. Detection of MMTV env gene-like sequences in DNA from human cell lines in culture

	Human Cell Lines		MMTV <u>env</u> gene sequence
5	MC-7	(breast carcinoma)	positive
	T47-D	" "	negative
	BT-20	" "	negative
	MDA-MB-231	" "	negative
	ZR-75-1	" "	negative
10	SK-BR 3	" "	negative
	BT474	" "	negative
	ED	" "	positive
	MCF-10	(normal breast)	negative
	HB-447	" "	negative
15	HL-60	(promyelocytic leukemia)	negative
	K562	(erythroleukemia)	negative
	Jurkat	(T cell leukemia)	negative
	Hep 6-2	(hepatoma)	negative

The nested polymerase reaction was used in several instances to increase sensitivity and specificity, thus reducing the probability of false positives. In Fig. 2, results of a representative nested reaction are shown using primers 1 and 4 in the first reaction (Fig. 2A) and 2 and 3 for the 2nd reaction. The specificity of the reaction can be seen in the 2nd amplification (Fig. 2B).

To study a large number of samples and to be able to perform archival studies, PCR of paraffin-embedded tissue sections was also carried out. Primers 2 and 3 were used to amplify a 250 bp sequence within the 660 bp stretch when DNA was extracted from paraffin-embedded tissue sections since larger size sequences are difficult to amplify after fixation. Tumor DNA was amplified (Fig. 3A, lanes 2-5) whereas normal breast DNA was not (Fig. 3A, lane 1). The identification of

this 250 bp sequence with the MMTV-like env gene was confirmed by hybridization with an internal probe (primer 2a) as shown in Fig. 3B. Using this procedure we have analyzed 151 breast cancer samples and found
5 that 60 (39.7%) possess the 250 bp sequence. Of the 27 normal breast samples obtained from reduction mammoplasties assayed by this procedure, one was positive (3.7%). These results, in conjunction with those obtained from lymphocytes and from normal breast
10 tissue of patients whose breast cancer was PCR positive, indicate that MMTV-like sequences are present in a significant number of human breast cancer DNA which cannot be explained by DNA polymorphism.

15 6.2.3. Cloning and Sequencing of the
MMTV-Like Env Gene Sequences

To find out whether there was homology to MMTV env gene throughout the whole 660 bp stretch, the product of the PCR from 8 different tumors was cloned and sequenced. In Fig. 4 the sequence of different clones
20 comprising around 600 bp are represented, as aligned to the MMTV env gene sequence of the GR and BR6 strains (Redmon, S. and Dickson, C., 1983, EMBO J. 2:125-131). This domain of the env gene in the GR strain is 100% homologous to the C₃H strain and 98% to the BR6 strain
25 (Majors, I.E. and Varmus, H.E., 1983, J. Virol. 47:495-504; Moore, R. et al., 1987, J. Virol. 61:480-490). Evaluation of the clones indicated that homology to MMTV env gene varied from 95% to 99%. Another seven clones comprising only 250 bp were also sequenced.
30 Homology to MMTV env gene varied from 95% to 99% (data not shown). When compared to the human endogenous provirus HERV-K10, the homology of all the clones was less than 15%. When compared against all known viral and human genes (more than 130,000 entries) using the
35 lBl MacVector GenBank and GCG programs, the highest homology recorded was 18%.

6.2.4. Southern Blot Analysis Using Cloned Sequences

To investigate whether the env gene-like sequences were present in human DNA, Southern blot hybridization was performed using the cloned sequence as probe. DNAs from normal breast tissues, env positive or negative breast tumors, tumors other than breast and breast cancer cell lines were restricted with EcoRI and in some instances with PstI, BglII or KpnI. EcoRI is a frequent cutter restriction enzyme that digests MMTV proviral DNA between env and pol genes. Four different cloned 660 bp sequences were used as probes after labeling with ³²P by random prime-labeling. Results of some of the Southern blot hybridization experiments are shown in Fig. 5. They reveal the presence of a labeled restriction fragment migrating at approximately 7-8 kb in breast cancer DNA, in ED and two fragments in MCF-7 cells. Different restriction patterns were observed with the other three enzymes. The 660 bp sequence was absent in 10 normal tissues, 10 fibroadenomas and 10 tumors from other tissues. It is important to emphasize that hybridization conditions for these experiments were stringent (as described in Section 6.1) to avoid interference with endogenous sequences that might interact with the probes.

7. Example: Detection of a Retrovirus Proviral Fragment in Human Breast Cancer Cells and Tissues

7.1. Materials and Methods

To detect longer retrovirus proviral fragments in breast cancer samples, DNA was extracted from breast cancer carcinoma tissue samples as described above in Section 6.1. Two rounds of long PCR was performed on the DNA primers 5L (SEQ ID NO:11) and LTR3 (SEQ ID NO:19). The primer 5L contains nucleotides 7370-7395 of the MMTV BR6 genome (5'-3': CCAGATCGCCTTTAAGAAGG) (SEQ ID NO:11) and primer LTR3 contains nucleotides

9918-9927 of the MMTV BR6 genome (5'-3':
CGAACAGACACAAAGCGACG) (SEQ ID NO:19). Long PCR was
performed using protocols described by the manufacturer
(Perkin Elmer, Foster City, CA). The amplified
5 retroviral fragment isolated from the breast cancer
sample was cloned into the TA cloning vector
(Invitrogen) and automated sequencing was performed
as described in Section 6.1.

7.2 Results

10 An approximately 2.6 kb retroviral fragment
containing 1,228 bp of the 3' LTR sequence and 1,336 bp
of the env gene sequence of a potential provirus was
detected in a human breast carcinoma tissue sample by
the long PCR technique using the 5L and LTR3 primers.
15 The sequence of this retroviral fragment is shown in
Fig. 9. (SEQ ID NO:20).

When compared with the two strains of MMTV C3H
and BR6, the sequence homology was 90.8% and 90.7%,
respectively, over the MMTV genomic fragment from
20 nucleotides 7370-9937. When compared with the
endogenous retroviral sequences (HUMERKA), sequence
homology was only 58% in 36 bp and 71% in 74 bp.

8. Discussion

Search for virus-related sequences in human breast
25 cancer has been hampered by great variation reported
in previous studies, by the presence of endogenous
retroviral sequences in human DNA and by the lack of
sensitivity of the methods employed. The studies
reported herein circumvent these deficiencies by
30 focusing on sequences with low homology to human
endogenous retroviruses, by investigating a large
number of tumors and several types of controls and
by using the most sensitive technology presently
available.

35 The results indicate that unique MMTV env gene
sequences were present in 38.5% of the breast cancer

samples analyzed and 39.7% of archival samples of breast cancer and that these sequences were absent in normal tissues including lymphocytes from patients with positive breast cancer and in cancers other than breast. Normal breast tissue and fibroadenomas had a low frequency (1.8 to 6.9%) of positive results. When cloned and sequenced, the sequences were found to be highly homologous to MMTV env gene, but not to the endogenous retroviral sequences. Furthermore, experiments in which the cloned amplified sequences were used for hybridization with DNA from breast cancer or normal tissues revealed that homologous DNA was only present in breast cancer DNA. The results also indicate that a human breast carcinoma sample contained an about 2.6 kb MMTV-like fragment comprised of 1,336 bp of the env gene and 1,228 bp of the 3' LTR.

The detection of MMTV env gene sequences in two fibroadenomas out of 29 and in two normal breast tissue samples out of 107 samples is of uncertain significance. Although such results could potentially be artifactual, and thus may represent false positives, they may alternatively indicate the presence of histologically unrecognized cells that were or will be neoplastic.

Ninety percent (90%) of the breast cancers tested were invasive ductal carcinomas, which reflects the prevalence of this type of neoplasm. Most patients were node-positive which is probably artifactual since it was necessary that tumor size be sufficiently large to provide an aliquot for research and tumor size correlates with node positivity.

It is unlikely that differences in homology between MMTV env gene and the cloned human sequences are generated by errors committed by the Taq polymerase. It has been estimated that the rate of nucleotide misincorporation is 1×10^{-5} per cycle (Ehrlich et al, 1991, Science 252:1643-1651) and

therefore, only a total of 0.32 nucleotides misincorporated should be expected in 660 bp after 50 cycles. The differences in homology between clones from different patients is likely to represent
5 heterogeneity of the env gene.

In contrast to earlier, ambiguous data associating MMTV-like sequences with human breast cancer, we have clearly demonstrated the existence of such sequences in breast cancer cells which cannot be explained by any
10 known human endogenous retroviral sequence. Our data do not support the results of earlier studies which indicated that, as in the mouse, MMTV-like sequences were found in lymphocytes from two patients with breast cancer (Crepin, M. et al., 1984, Biochem. Biophys. Res.
15 Comm. 118:324-331). The absence of MMTV env-like sequences in lymphocytes could reflect the fate of a unique lymphocyte subset over decades between initial encounter and the appearance of clinical breast cancer; alternatively, the human disease may differ from the
20 mouse model. Results from attempts to identify unique MMTV-like pol gene sequences have shown that they cannot be distinguished from the reverse transcriptase sequences of endogenous retroviruses (Deen, K.C. and Sweet, R.W., 1986, J. Virol. 57:422-432).

25 The origin of the MMTV env gene-like and 3' LTR-like sequences found in tumor DNA could be the result of integrated MMTV-like sequences from a human mammary tumor virus. Polymorphism of endogenous retroviral sequences is conceivable but can be ruled out because
30 these sequences were not detected in lymphocytes from the positive patients, in sections of the cancerous breast from which abnormal cells were absent, or in normal breast tissue from patients with MMTV env-like positive tumors. Recombination during tumorigenesis
35 between endogenous sequences to resemble the MMTV env genes seems highly unlikely since no known gene or viral sequence is more than 18% homologous to the

660 bp sequence. The longer about 2.6 kb MMTV-like fragment detected in a human breast carcinoma had minimal homology (58% in 36 bp and 71% in 74 bp) to endogenous human retroviral sequences. Thus, the most conservative interpretation is that our findings represent exogenous sequences from an agent similar to MMTV. Recombination between endogenous and exogenous env gene sequences are known to accelerate the development of malignancies in mice (DiFronzo, N.L. and Holland, C.A., 1993, J. Virol. 67:3763-3770). Whether the MMTV-like sequences belong to an entire acquired provirus or to an exogenous fragment integrated into endogenous sequences, is presently not known. Experiments are in progress to distinguish between these possibilities.

Several genetic alterations have been identified in human breast cancer that can be useful as markers for prevention, detection or prognosis (reviewed in Runnenbaum, I. et al., 1991, Proc. Natl. Acad. Sci. USA 88:10657-10661). The BRCA1 and BRCA2 genes have recently been described. They account for at least 5% of breast cancer and are related to familial breast cancer (Miki, Y. et al., 1994, Science 266:66-71; Wooster, R. et al., 1994, Science 265:2088-2090). We have primary evidence that familial clustering of the MMTV env gene-like sequences occurs, accounting for an even higher percentage of cancers in affected families (Holland et al. 1994, Proc. Am. Assoc. Cancer Res 35:218). The presence of MMTV-like sequences may be correlated with special clinical disease status, may provide another potential molecular marker, and may distinguish a subset of human breast cancer for which viral etiology is tenable. This has implications for epidemiology, therapy and prevention.

Various publications are cited herein, the contents of which are hereby incorporated by reference in their entireties.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: HOLLAND, JAMES

5 (ii) TITLE OF THE INVENTION: DETECTION OF MAMMARY TUMOR VIRUS-LIKE
SEQUENCES IN HUMAN BREAST CANCER

1 (iii) NUMBER OF SEQUENCES: 20

(iv) CORRESPONDENCE ADDRESS:

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(F) ZIP: 10112-0228

15 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 1.5

20 (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: NOT YET ASSIGNED
(B) FILING DATE: 08-NOV-1996
(C) CLASSIFICATION:

25 (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER 08/555,394
(B) FILING DATE: 09-NOV-1995

30 (viii) ATTORNEY/AGENT INFORMATION:

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(A) TELEPHONE: 212-408-2628
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35 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

45 (vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCTCACTGCC AGATC

15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGGAATTCCT CACTGCCAGA TC

22

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCTCACTGCC AGATCGCCT

19

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TACATCTGCC TGTGTTAC

18

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
CCTACATCTG CCTGTGTTAC

20

10 (2) INFORMATION FOR SEQ ID NO:6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
20 (vi) ORIGINAL SOURCE:
(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
CCGCCATACG TGCTG

15

25 (2) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
ATCTGTGGCA TACCT

15

40 (2) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGGAATTCAT CTGTGGCATA CCT

23

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATCTGTGGCA TACCTAAAGG

20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GAATCGCTTG GCTCG

15

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCAGATCGCC TTTAAGAAGG

20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TACAGGTAGC AGCACGTATG

20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Leu	Lys	Arg	Pro	Gly	Phe	Gln	Glu	His	Glu	Met	Ile
1				5					10		

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Gly	Leu	Pro	His	Leu	Ile	Asp	Ile	Glu	Lys	Arg	Gly
1				5					10		

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Thr Asn Cys Leu Asp Ser Ser Ala Tyr Asp Thr Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:16:

10

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Asp Ile Gly Asp Glu Pro Trp Phe Asp Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:17:

25

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 662 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

35	TCCTCACTGC	CAGATCGCCT	TTAAGAAGGA	CGCCTTCTGG	GAGGGAGACG	AGTCTGCTCC	60
	TCCACGGTGG	TTGCCTTGCG	CCTTCCCTGA	CCAAGGGGTG	AGTTTTTCTC	CAAAAGGGGC	120
	CCTTGGGGTA	CTTGGGGATT	TCTCCCTTCC	CTCGCCTAGT	GATAGTCAGT	CAGATCAGAT	180
	TAAAAGCAAA	AAGGATCTAT	TTGGAAATTA	TACTCCCCCA	GTCAATAAAG	AGGTTTCATCG	240
	ATGGTATGAA	GCAGGATGGG	TAGAACCTAC	ATGGTTCTGG	GAAAATTCTC	CTAAGGATCC	300
40	CAATGATAGA	GATTTTACTG	CTCTAGTTCC	CATACAGAAT	TGTTTCGCTT	AGTTGCAGCC	360
	TCAAGATATC	TTATTCTCAA	AAGGCAGGAT	TTCAGGAACA	TGAGATGATT	CCTACATCTC	420
	TGTGTTACTT	ACCCTTATGT	CATATTATTA	GGATTACCTC	AGCTAATAGA	TATAGAGAAA	480
	GAGGATCTAC	TTTTCATATT	TCCTGTTCTT	CTTGATGATT	GACTAATTGT	TTAGATTCTT	540
	CTGCCTACGA	CTATGCAGCG	ATCATAGTCA	AGAGGCCGCC	ATACGTGCTG	CTACCTGTAG	600
45	ATATTGGTGA	TGAACCATGG	TTTGATGATT	CTGCCATTCA	AACCTTTAGG	TATGCCACAG	660
	AT						662

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 663 base pairs
(B) TYPE: nucleic acid

50

(C) STRANDEDNESS: single
(D) TOPOLOGY: lin ar

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

10	TCCTCACTGN CAGATCGCCT TTAAGAAGGA CGCCTTCTGG GAGGGAGACG AGTCTGCTCC	60
	TCCACGGTGG TTGACTTGCG CCTTCCCCTGA CCAGGGGGTG AGTTTTTCTC CAAAAGGGGC	120
	CCTTGGGTTA CTTTGGGATT TCTCCCTTCC CTCGCCTAGT GTAGATCAGT CAGATCAGAT	180
	TAAAAGCAAA AAGGATCTAT TTGGAAATTA TACTCCCCCT GTCAATAAAG AGGTTTCATCG	240
	ATGGTATGAA GCAGGATGGG TAGAACCTAC ATGGTTCTGG GAAAATTCTC CTAAGGATCC	300
15	CAATGATAGA GATTTTACTG CTCTAGTTCC CATAAGAAT TGTTTCGCTT AGTTGCAGCC	360
	TCAAGATATC TTATTCACAA AAGGCAGGAT TTCAAGAACA TGACATGAAT CCCTACATCT	420
	CTGTGTTACT TACCCTTATG CCANANTATT AGGATTACCT CAGCTAATAG ATATAGAGGA	480
	AGAGGATCTA CTTTTCATAT TTCCTGTTCT TCTTGTAGAT TGACTAATTG TTTAGATTCT	540
	TCTGCCTACG ACTATGCAGC GATCATAGTC AAGAGGCCGC CATACGTGCT GCTACCTGTA	600
20	GATATTGGTG ATGAACCATG GTTTGATGAN NCTGCCANTC AAACCTTTAG GTATNCCACA	660
	GAT	663

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGAACAGACA CAAACACACG

20

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2598 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

	CGAACAGACA	CAAACACACG	AGAGGTGAAT	GTTAGGACTG	TTGCAAGTTT	ACTCAAAAAA	60
	CAGCACTCTT	TTATATCATG	GTTTACATAA	GCATTTACAT	AAGACTTGGA	TAAGTTCCAA	120
5	AAGAACATAG	GAGAATAGAA	CACTCAGAGC	TTAGATCAAA	ACATTTGATA	CCAAACCAAG	180
	TCAGGAAACC	ACTTGTCTCA	CATCCTTGTT	TTAAGAACAG	TTTGTGACCC	TGAACCTACT	240
	TAAACCTTGG	GAACCGCAAN	GTTGGGCTCA	TAAAGGTTAT	CCATTATAGC	TCATGCCAAA	300
	ATTATCTGCA	GAAATGTGTT	CCTAATGTGC	TAGCCACTGC	CCCCTCCCTT	GGTATAATGA	360
	AAATCTTTCC	CCCAACGTTT	ATCCCACTCC	CCTAGATAAA	TATAATCATG	TACCTGTTGT	420
10	TTTATGTCGT	CTTTTTCTTC	CTGAGTTAAC	ACACACCAAG	GAGGTCTAGC	TCTGGCGAGT	480
	CTTTCACGAA	AGGGGAGGGA	TCTGTACAAC	ACTTTATAGC	CGTTGACTGT	GACCCACCTA	540
	TCGAAATTTA	AATCGTATCT	TCCTGTATAT	GGTAGCGGGG	CGTCTGTTGG	TCTGTAGATG	600
	TAAGTCCCGG	TTGCCACCAC	CTGTCTCCTA	TTTTGACAAG	CGTACTCCTC	TTCCTCCCTT	660
	TTACTTCTAG	GCCTGAGGCC	CTTAGTCCTT	GCACCTGTTT	TTCAACTGAG	GTTGAGCGTC	720
15	TCTTTCTATT	TTCTATTCCC	ATTTCTAACC	TTTGAATTTG	AGTAAATATA	GTGCTAAAAG	780
	ACAAAGATTC	ATTTCTTAAC	ATCATGATTA	ATAATCGACC	TATTGGATTG	GTCTTATTGG	840
	TAAAAATATA	ATTTTATAGC	AGCATTCTTA	TTTCTATTTC	TGAAGGACAA	AGTCGGGTGTG	900
	GCTTGTAANA	GGAANTTGGC	TGTGGTCCTT	GCCCCACGAG	GAAGGTGCGAG	TTCTCCGAAT	960
	TGTTTAGATT	GTAATCTTGC	ACAGAAGAGT	TATTAAGAAG	ATCAAGGGTG	AGAGCCCTGC	1020
20	GAGCACGAAC	CGCAACTTCC	CCCAATAGCC	CCAGGCAAAG	CAGAGCTATG	CCAAGTTTGC	1080
	AGCAGANAAT	GAGTATGTCT	TTGTCTGATG	GGCTCATCCG	CGTGCACGCA	GACGGGTCGT	1140
	CCTTGGTGCG	AAACAACCCC	TTGGCTGCTT	CTCTCCTAAG	TGTAGGACAC	TCTCGGGAGT	1200
	TCAACCATT	CTGCTGCAGG	CGCGGCATTT	CCCCCTTTTT	TCTTTTTTAA	AAGAAGCAGC	1260
	TTAAGATCTG	ACTGCACTTG	GTCAAGGCTC	TTGCGAAAGC	ACTGGAAAAT	AACGGGGAAA	1320
25	ATCATAAGTA	CTATGACCAA	AAGCAGGGCT	CCAATCCTA	TAAAAATGAA	ATATTGTGTT	1380
	CTAATCCAAT	GGATTTAAAG	CCTTTACTCC	ATTGGCNAAG	GANTGANCCA	ACCCCTGAGG	1440
	TCCCTGCGTT	CAAATTTTTT	TGCTCNTATC	CTAATCCAAT	TGGTAACCCC	GTTTNTTTTT	1500
	GAAACTCATG	TCTTCAAATG	CCCAATAAAT	GAGCCCTGGT	TCTTTCCCAG	CTCTCAGAAG	1560
	CATTATACGG	NANAGGTGTG	ACACAGCATA	AAATCATAAT	TTGCATGACA	CCTAGTGGAC	1620
30	ATTCTGGTCT	TTAAGTTTGC	CACATCTTGT	CCCAACTCTA	AAACTACTTC	TTCTAAAGCA	1680
	TTAAGTCTAG	CTTCAATTTT	TAAGTCTATT	ATTCTTTGTT	CAGATNAGGC	TAATGTAACA	1740
	TTTCTATGAA	GATTATTAAC	AAACGTAGCA	GTTTGCATCT	CCTTAACTAA	GGCAGTAGTA	1800
	GCTACAGCAA	AGGAAGTGAT	AATAGCAATT	AAAGCAGATA	TGCCCAGAAT	AATGGCAGCG	1860
	ACGAATCGCT	TAGCTCGAAT	TAAATCTGTG	GCATACCTAA	AGGTTTGAAT	GGCAGAATCA	1920
35	TCAAACCATG	GTTTCATCAC	AATATCTACA	GGTTACAACA	CATATGGCGG	CCCCTTGAAT	1980
	ATGAATCGCT	GCATATCCGT	NGGCAAAAAA	TCTAACCATT	ATTCTCTCTN	CCNAAAAACG	2040
	GGATTTGAAA	NTTATNCCCC	TTNCCCCNAA	CCCANACCGA	GGTACCCCAT	AATGNGGGGG	2100
	GTATCTANAA	NAGGGCATAG	GGGTAAGAAA	AACGGCAGAG	NGGGATCNTT	TATGTTTCNGG	2160
	AAATTTCNGG	TTTGGGAGAA	TAAGATTCTG	GAGGCTGCAA	ATTAAGGGAA	ACATTNTGTA	2220
40	TGGGGAATAG	AGCAGTAAAA	TCTCTATCAT	GGGGATCTTT	AGGGAGAATT	TTCCAGGAA	2280
	CCAAGTAGGT	TCNAACCCAT	CNTGCTTCAT	ACCATCGATG	AACNTCTTTA	TTGACAGGGG	2340
	GAGTATAATT	TCCAAATAGA	TCCTTTTTGT	TTTTAATCTG	ATCTGACTGA	TCTACACTAG	2400
	GCGGGGGAAG	GGAGAAATCC	CAAAGTAACC	CAAGGGCCCC	TTTTGGAGAA	AAACTCACCC	2460
	CCTGGTCAGG	GAAGGCGCAA	GGCAACCACC	GTGGAGGAGC	AGACTCGTCT	CCCTCCCGAG	2520
45	AGGCGTCCTT	CTTAAAGGCG	ATCTGGAGGA	GCAGACTCGT	CTCCCTCCCA	GAAGGCGTCC	2580
	TTCTTAAAGG	CGATCTGG					2598

Claims

- 1 1. A composition comprising an oligonucleotide primer
2 which may be used to detect the presence of a
3 nucleic acid molecule which (i) hybridizes to the
4 env gene of a mouse mammary tumor virus; (ii) is
5 present in at least 38 percent of DNA samples
6 prepared from breast cancer tissue of different
7 human subjects; and (iii) hybridizes to less than
8 7 percent of DNA samples prepared from tissues
9 other than breast cancer tissue from different
10 human subjects.
- 1 2. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 CCTCACTGCCAGATC (SEQ ID NO:1).
- 1 3. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 GGGAATTCTCACTGCCAGATC (SEQ ID NO:2).
- 1 4. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 CCTCACTGCCAGATCGCCT (SEQ ID NO:3).
- 1 5. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 TACATCTGCCTGTGTTAC (SEQ ID NO:4).
- 1 6. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 CCTACATCTGCCTGTGTTAC (SEQ ID NO:5).
- 1 7. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 CCGCCATACGTGCTG (SEQ ID NO:6).

- 1 8. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 ATCTGTGGCATACCT (SEQ ID NO:7).
- 1 9. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 GGAATTCATCTGTGGCATACCT (SEQ ID NO:8).
- 1 10. The composition of claim 1, wherein the
2 oligonucleotide primer comprises a sequence
3 selected from the group consisting of
4 ATCTGTGGCATACCTAAAGG (SEQ ID NO:9);
5 GAATCGCTTGGCTCG (SEQ ID NO:10);
6 CCAGATCGCCTTTAAGAAGG (SEQ ID NO:11); and
7 TACAGGTAGCAGCACGTATG (SEQ ID NO:12).
- 1 11. An essentially purified peptide encoded by a
2 nucleic acid molecule which (i) hybridizes to
3 a gene of MMTV; (ii) is present in at least
4 20 percent of DNA samples prepared from breast
5 cancer tissue of different human subjects; and
6 (iii) is present in less than 5 percent of DNA
7 samples prepared from tissues other than breast
8 cancer tissue from different human subjects.
- 1 12. An antibody which specifically binds to the
2 peptide of claim 11.
- 1 13. The peptide according to claim 11 which comprises
2 the amino acid sequence LKRPGFQEHEMI (SEQ ID
3 NO:13).
- 1 14. An antibody which specifically binds to the
2 peptide of claim 13.
- 1 15. The peptide according to claim 11 which comprises
2 the amino acid sequence GLPHLIDIEKRG (SEQ ID NO:14).

- 1 16. A method of diagnosing breast cancer in a human
2 subject, comprising detecting the presence of a
3 peptide encoded by a nucleic acid molecule which
4 (i) hybridizes to the env gene of 3' LTR of a
5 mouse mammary tumor virus; (ii) is present in at
6 least 20 percent of DNA samples prepared from
7 breast cancer tissue of different human subjects;
8 and (iii) is present in less than 5 percent of DNA
9 samples prepared from tissues other than breast
10 cancer tissue from different human subjects.
- 1 17. The method according to claim 16, wherein the
2 presence of a peptide comprising the amino acid
3 sequence LKRPGFQEHEMI (SEQ ID NO:13) is detected
4 by the binding of an antibody specific to the
5 peptide.
- 1 18. The method according to claim 16, wherein the
2 presence of a peptide comprising the amino acid
3 sequence GLPHLIDIEKRG (SEQ ID NO:14) is detected
4 by the binding of an antibody specific to the
5 peptide.
- 1 19. The method according to claim 16, wherein the
2 presence of a peptide comprising the amino acid
3 sequence TNCLDSSAYDTA (SEQ ID NO:15) is detected
4 by the binding of an antibody specific to the
5 peptide.
- 1 20. The method according to claim 16, wherein the
2 presence of a peptide comprising the amino acid
3 sequence DIGDEPWFDD (SEQ ID NO:16) is detected by
4 the binding of an antibody specific to the
5 peptide.
- 1 21. A composition comprising an oligonucleotide primer
2 which may be used to detect the presence of a

3 nucleic acid molecule which (i) hybridizes to a
4 nucleic acid comprised of a sequence selected from
5 the group consisting of the env gene and the 3'
6 LTR of a mouse mammary tumor virus; (ii) is
7 present in a substantial percentage of DNA samples
8 prepared from breast cancer tissue of different
9 human subjects; and (iii) hybridizes to less than
10 5 percent of DNA samples prepared from tissues
11 other than breast cancer tissue from different
12 human subjects.

1 22. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 CCAGATCGCCTTTAAGAAGG (SEQ ID NO:11).

1 23. The composition of claim 1, wherein the
2 oligonucleotide primer comprises the sequence
3 CGAACAGACACAAACACACG (SEQ ID NO:19).

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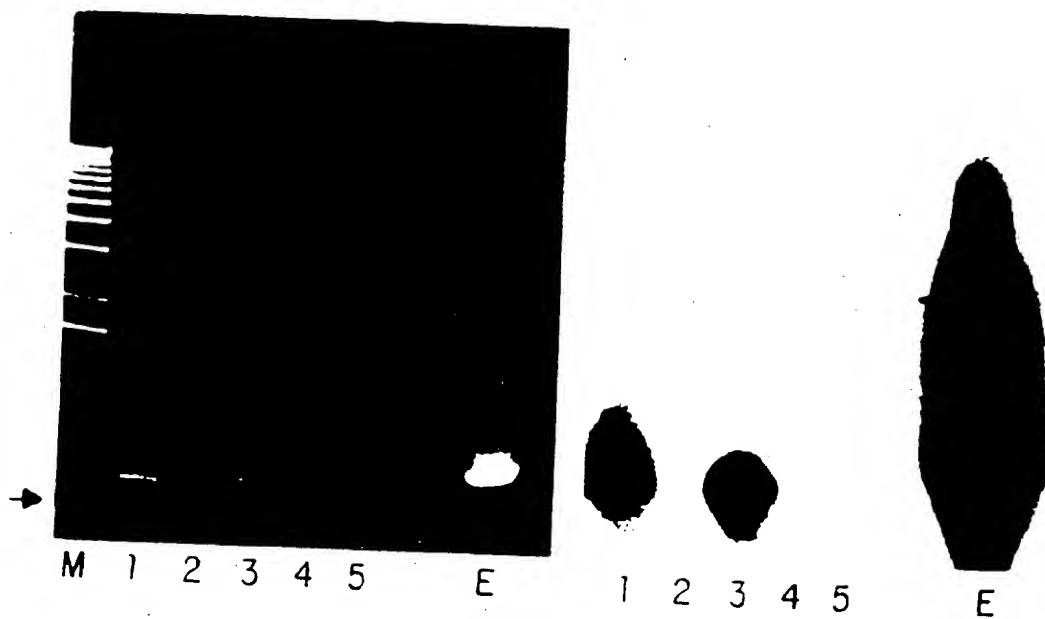


FIG. 1A

FIG. 1B

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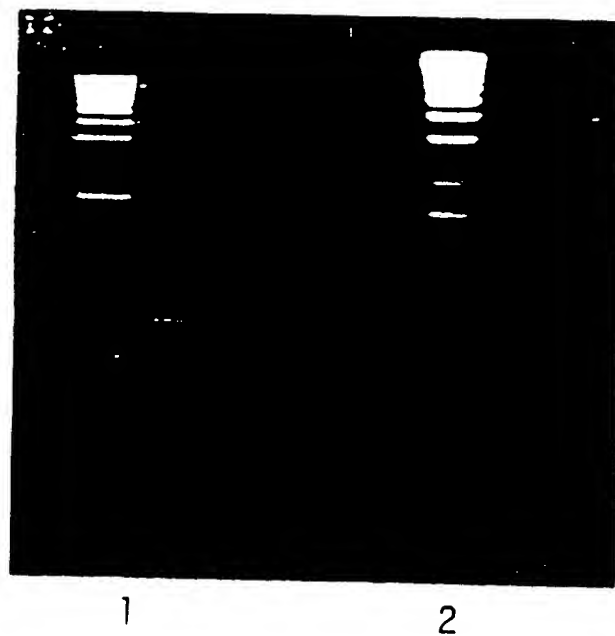


FIG.2A



FIG.2B

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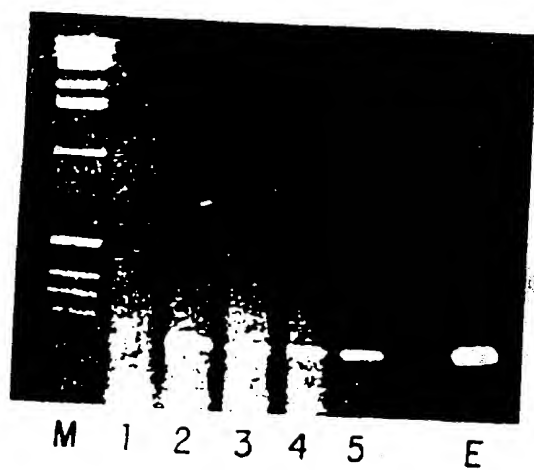


FIG.3A

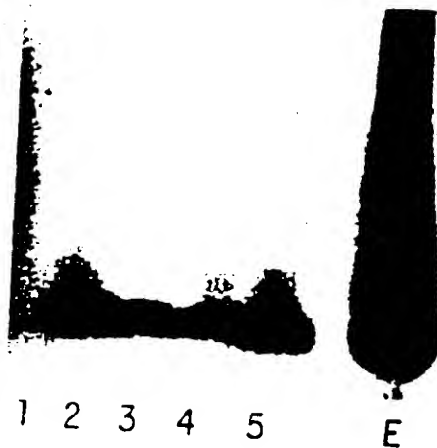


FIG.3B

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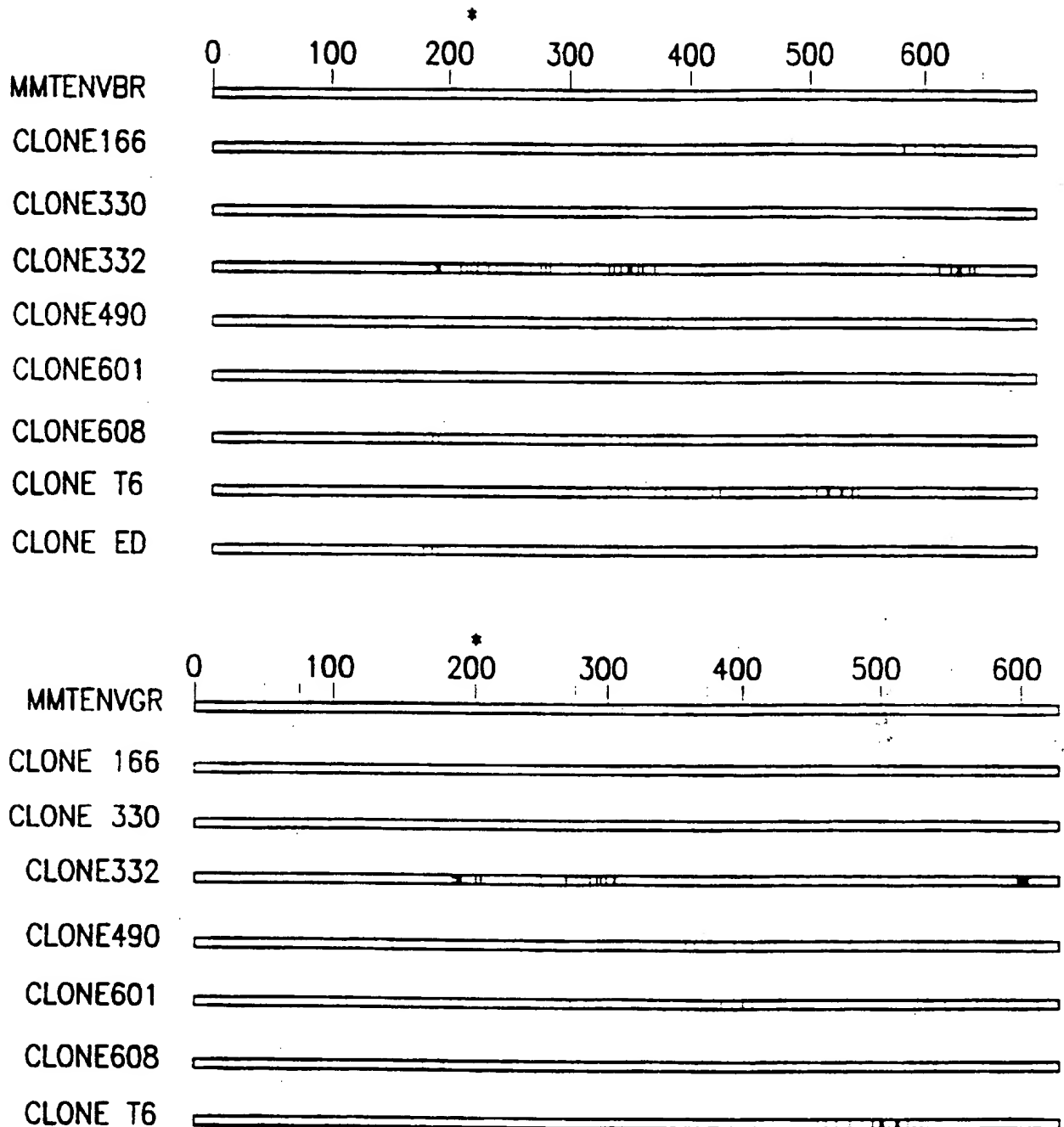


FIG.4

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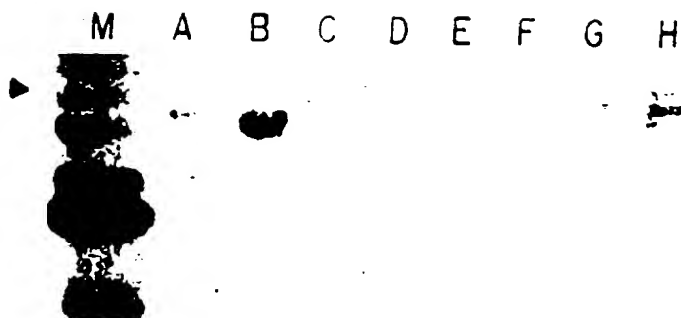


FIG.5

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A B C D E F G H



FIG.6

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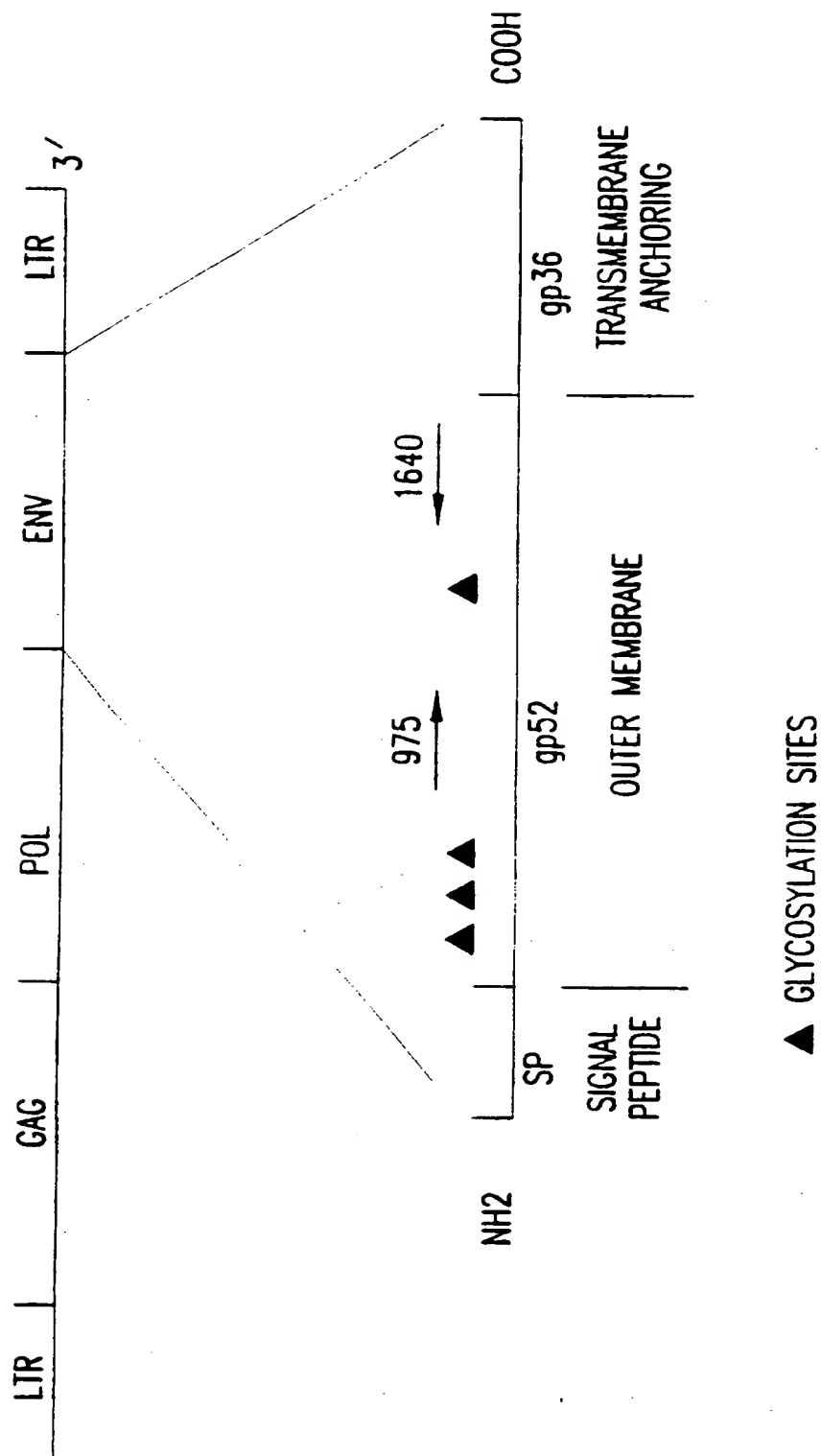


FIG. 7

MMTENV 974 TCCTCACTGCCAGATCGCCTTTAAGAAGGACGCCTTCTGGGAGGGAGACG 1023
|||||:|||||
MS1627 1 TCCTCACTGNCAGATCGCCTTTAAGAAGGACGCCTTCTGGGAGGGAGACG 50

MMTENV1024 AGTCTGCTCCTCCACGGTGGTTGCCCTTGCGCCTTCCCTGACCAAGGGTG 1073
|||||
MS1627 51 AGTCTGCTCCTCCACGGTGGTTGACTTGCGCCTTCCCTGACCAAGGGTG 100

MMTENV1074 AGTTTTTCTCCAAAAGGGCCCTTGGGTACTTTGGGATTTCTCCCTTCC 1123
|||||
MS1627 101 AGTTTTTCTCCAAAAGGGCCCTTGGGTACTTTGGGATTTCTCCCTTCC 150

MMTENV1124 CTCGCCCTAGTGTAGATCAGTCAGATCAGATTAAAGCAAAAGGATCTAT 1173
|||||
MS1627 151 CTCGCCCTAGTGTAGATCAGTCAGATCAGATTAAAGCAAAAGGATCTAT 200

MMTENV1174 TTGGAAATTATACTCCCCCAGTCAATAAAGAGGTTTCATCGATGGTATGAA 1223
|||||
MS1627 201 TTGGAAATTATACTCCCCCTGTCAATAAAGAGGTTTCATCGATGGTATGAA 250

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FIG. 8A

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MMTENV1224 GCAGGATGGGTAGAACCTACATGGTTCTGGGAAATTCTCCTAAGGATCC 1273
|||||
MS1627 251 GCAGGATGGGTAGAACCTACATGGTTCTGGGAAATTCTCCTAAGGATCC 300
|||||

MMTENV1274 CAATGATAGAGATTTTACTGCTCTAGTTCCCATACAGAAATGTTTCGCT 1323
|||||
MS1627 301 CAATGATAGAGATTTTACTGCTCTAGTTCCCATACAGAAATGTTTCGCT 350
|||||

MMTENV1324 TAGTTGCAGCCTCAAGATATCTTATTCTCAAAGGCAGGATTTCAGGAA 1373
|||||
MS1627 351 TAGTTGCAGCCTCAAGATATCTTATTCTCAAAGGCAGGATTTCAGGAA 400
|||||

MMTENV1374 CATGAGATG-ATCCCTACATCTCTGTGTACTTACCCTTATGTCATATT 1423
|||||
MS1627 401 CATGACATGAATCCCTACATCTCTGTGTACTTACCCTTATGCCANANT 450
||-|-|

MMTENV1424 ATTAGGATTACCTCAGCTAATAGATATAGAGAAAGAGGATCTACTTTC 1473
|||||
MS1627 451 ATTAGGATTACCTCAGCTAATAGATATAGAGAAAGAGGATCTACTTTC 500
|||||

FIG. 8B

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MMTENV1474 ATATTTCCCTGTTCTTCTTGTAGATTGACTAATTGTTTAGATTCTTCTGCC 1523
|||||
MS1627 501 ATATTTCCCTGTTCTTCTTGTAGATTGACTAATTGTTTAGATTCTTCTGCC 550

MMTENV1524 TACGACTATGCAGCGATCATAGTCAAGAGGCCGCCATACGTGCTGTACC 1573
|||||
MS1627 551 TACGACTATGCAGCGATCATAGTCAAGAGGCCGCCATACGTGCTGTACC 600

MMTENV1574 TGTAGATATTGGTGATGAACCATGGTTTGATGATCTCTGCCATTCAAACCT 1623
|||||
MS1627 601 TGTAGATATTGGTGATGAACCATGGTTTGATGANNCTGCCANTCAAACCT 650
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MMTENV1624 TTAGGTATGCCACA GAT 1640
|||||
MS1627 651 TTAGGTATNCCACA GAT 667

FIG. 8C

CGAACAGACACAAACACACGAGAGGTGAATGTTAGGACTGTTGCAAGTTTA
CTCAAAAAACAGCACTCTTTTATATCATGGTTTACATAAGCATTACATAAGA
CTTGGATAAGTTCCAAAAGAACATAGGAGAATAGAACACTCAGAGCTTAGAT
CAAAACATTTGATACCAAACCAAGTCAGGAAACCACTTGTCTCACATCCTTG
TTTTAAGAACAGTTTGTGACCCTGAACCTTACTTAAACCTTGCGAACCGCAAN
GTTGGGCTCATAAAGGTTATCCATTATAGCTCATGCCAAAATTATCTGCAGA
AATGTGTTCCTAATTGTCTAGCCACTGCCCCCTCCCTTGGTATAATGAAAT
CTTCCCCCAACGTTTCATCCCACTCCCTTAGATAAATATAATCATGTACCTGT
TGTTTTATGTCGTCTTTTTCTTCTGAGTTAACACACACCAAGGAGGTCTAGC
TCTGGCGAGTCTTTCACGAAAGGGGAGGGATCTGTACAACACTTTATAGCC
GTTGACTGTGACCCACCTATCGAAATTTAAATCGTATCTTCTGTATATGGTA
GCGGGGCGTCTGTTGGTCTGTAGATGTAAGTCCCGGTTGCCACCACCTGTC
TCCTATTTTGACAAGCGTACTCCTCTTTCCCTTTTTAGTTCTAGGCCTGAGG
CCCTTAGTCCTTGCACTGTTCTTCAACTGAGGTTGAGCGTCTCTTTCTATTT
TCTATTCCCATTTCTAACCTTTGAATTTGAGTAAATATAGTGCTAAAAGACAA
AGATTCATTTCTTAACATCATGATTAATAATCGACCTATTGGATTGGTCTTATT
GGTAAAAATATAATTTTTAGCAAGCATTCTTATTTCTATTTCTGAAGGACAAA
GTCCGTGTGGCTTGTAANAGGAANTTGGCTGTGGTCTTGCCCCACGAGGA
AGGTGAGTTCTCCGAATTGTTTAGATTGTAATCTTGCACAGAAGAGTTATTA
AAAGAATCAAGGGTGAGAGCCCTGCGAGCACGAACCGCAACTTCCCCCAAT
AGCCCCAGGCAAGCAGAGCTATGCCAAGTTTGCAGCAGANAATGAGTATG
TCTTTGTCTGATGGGCTCATCCGCGTGCACGCAGACGGGTCGTCTTGGTG
GGAAACAACCCCTTGGCTGCTTCTCTCCTAAGTGTAGGACACTCTCGGGAG
TTCAACCATTTCTGCTGCAGGCGCGGCATTTCCCCCTTTTTCTTTTTAAAA
GAAGCACGTTAAGATCTGACTGCACTTGGTCAAGGCTCTTCGCAAAGCACT
GGAAAATAACGGGGAAAATCATAAGTACTATGACCAAAAGCAGGGCTCCAA
CTCCTATAAAAATGAAATATTGTGTTCTAATCCAATGGATTTAAAGCCTTTAC
TCCATTGGCNAAGGANTGANCCAACCCCTGAGGTCCTGCGTTCAAATTTTT
TTGCTCNTATCCTAATCCAATTGGTAACCCCGTTTNTTTTTGAAACTCATGTC
TTCAAATGCCCAATAAATGAGCCCTGGTCTTTCCAGCTCTCAGAAGCATT
ATACGGNANAGGTGTGACACAGCATAAAATCATAATTTGCATGACACCTAGT
GGACATTCGGTCTTTAAGTTTGCCACATCTTGCCCAACTCTAAAAC TACTT
CTTCTAAAGCATTAAAGTCTAGCTTTCAATTTTAAGTCTATTATTCTTTGTTTCA
ATNAGGCTAATGTAACATTTCTATGAAGATTATTAACAAACGTAGCAGTTTGC
ATCTCCTTAAC TAAGGCAGTAGTAGCTACAGCAAAGGAAGTGATAATAGCAA
TTAAAGCAGATATGCCCAGAATAATGGCAGCGACGAATCGCTTAGCTCGAAT
TAAATCTGTGGCATACCTAAAGGTTTGAATGGCAGAATCATCAAACCATGGT
TCATCACCAATATCTACAGGTTACAACACATATGGCGGCCCTTGAATATGA
ATCGCTGCATATCCGTNGGCAAAAAATCTAACCATTATTCCTCCTNCCNAAA
AACGGGATTTGAAANTTATNCCCCCTNCCCCNAACCCANACCGAGGTACCC
CATAATGNGGGGGGTATCTANAANAGGGCATAGGGGTAAGAAAAACGGCA
GAGNGGGATCNTTTATGTTTNGGAAATTCNGGGTTTGGGAGAATAAGATTCT
GGAGGCTGCAAAATTAAGGGAAACATTNTGTATGGGGAATAGAGCAGTAAAA
TCTCTATCATGGGGATCTTTAGGGAGAATTTTCCAGGAACCAAGTAGGTTT
NAACCCATCNTGCTTCATACCATCGATGAACNTCTTTATTGACAGGGGGAGT
ATAATTTCCAAATAGATCCTTTTTGTTTTAATCTGATCTGACTGATCTACACT
AGGCGGGGGAAGGGAGAAATCCCAAAGTAACCCAAGGGCCCCCTTTTGGAG
AAAACTCACCCCTGGTCAGGGAAGGCGCAAGGCAACCACCGTGGAGGA
GCAGACTCGTCTCCCTCCCAAGGCGTCTTCTTAAAGGCGATCTGGAGG
AGCAGACTCGTCTCCCTCCCAAGGCGTCTTCTTAAAGGCGATCTGG

FIG.9

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17877**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet.

US CL : 435/6, 5, 91.2, 7.1, 7.2; 536 23.1, 24.3, 24.33; 530/388.1, 300

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 5, 91.2, 7.1, 7.2; 536 23.1, 24.3, 24.33; 530/388.1, 300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	REDMOND et al. Sequence and expression of the mouse mammary tumour virus env gene. The EMBO Journal. 1983, Volume 2, Number 1, pages 125-131. See entire document.	1-20
A	FAFF et al., Retrovirus-like particles from the human T47D cell lines are related to mouse mammary tumour virus and are of human endogenous origin. Journal of General Virology. 21 May 1992, Volume 73, pages 1087-1097. See abstract.	1-20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

04 FEBRUARY 1997

Date of mailing of the international search report

18 MAR 1997

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Authorized officer

DIANNE REES

Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17877

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CREPIN et al. Sequences Related to Mouse Mammary Tumor Virus Genome in Tumor Cells and Lymphocytes from Patients with Breast Cancer. Biochemical and Biophysical Research Communications. 13 January 1984, Volume 118, Number 1, pages 324-331. See entire document.	1-20
A	MESA-TEJADA et al. Detection in human breast carcinomas of an antigen immunologically related to a group-specific antigen of mouse mammary tumor virus. Proceedings of the National Academy of Sciences, USA. March 1978, Volume 75, Number 3, pages 1529-1533.	1-20*

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A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C12Q 1/68, 1/70; C12P 19/34; C07H 21/02, 21/04; G01N 33/53; C07K 15/28; 5/00

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, BIOTECHABS, BIOTECHDS, CABA, CAPLUS, CANCERLIT, DGENE, DRUGU, EMBASES, MEDLINE, USPATFULL, TOXLIT, TOXLINE, JAPIO, WPIDS

search terms: MMTV, mouse mammary tumor virus, PCR, hybridization, antibodies, immunoassays, Westerns, searched SEQ. ID. Nos.

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